AOP ID and Title:

SNAPSHOT

Created at: 2017-12-04 05:24

AOP 149: Peptide Oxidation Leading to Hypertension

Short Title: Hypertension

Authors

British American Tobacco: Frazer Lowe (Frazer_Lowe@bat.com); Linsey Haswell; Marianna Gaca

Philip Morris International: Karsta Luettich; Marja Talikka ; Julia Hoeng

Selventa: Vy Hoang (vhoang@selventa.com)

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.50	Included in OECD Work Plan

Abstract

Hypertension is a cardiovascular risk factor that has a profound influence on cardiovascular morbidity and mortality. While good progress has been made in terms of identifying and managing this risk factor for patient care, methods to assess the potential of chemical compounds to induce hypertension, or to assess the efficacy of consumer products (e.g. e-cigarettes, tobacco heating products) targeted at reducing disease burden remain largely limited to epidemiological associations and *in vivo* studies.

Here we present the supporting information on an AOP describing how vascular endothelial peptide oxidation leads to hypertension via perturbation of endothelial nitric oxide (NO) bioavailability. The molecular initiating event is oxidation of amino acid (AA) residues on critical peptides of the NO pathway, notably protein kinase B (AKT), guanosine triphosphate cyclohydrolase-1 (GTPCH-1), endothelial nitric oxide synthase (eNOS), and also the cellular ROS scavenger; glutathione. Oxidation of the enzymic components of the pathway lead to reduced expression of the phosphorylated proteins, and protein loss via proteasomal degradation. Oxidation of reduced glutathione to GSSG promotes bonding of GSSG to critical AA residues on eNOS, and the reduced expression of GTPCH-1 reduces bioavailability of tetrahydrobiopterin (BH4), both of which lead to uncoupling of eNOS (reduced NO production, increased superoxide production). The combination of these molecular events lead to reduced bioavailabilty of NO, which in turn reduces the potential for vasodilation and shifts the balance of vascular tone towards vasoconstriction. Repeated perturbation of this pathway via chronic exposure to toxicants, ultimately increases vascular resistance and contributes towards the development of hypertension.

The evidence supporting the AOP is strong from the molecular level up to impaired vasodilation, however there is a knowledge gap concerning the magnitude of contribution of this mechanism towards the development of hypertension, given the complexity of the disorder. Further AOPs are likely required to characterise the contribution of competing/promoting mechanisms which alter vascular tone towards chronic vasoconstriction.

With respect to the regulatory context, the AOP is of relevance to the risk assessment of airborne pollutants and other chronically inhaled toxicants which affect vascular endothelial cells. Additionally, acute and chronic exposure to aerosols generated by alternative tobacco products and nicotine delivery devices are poorly understood with respect to their impact on cardiovascular disease risk. Reliable *in vitro* models and human clinical biomarkers would help to inform regulatory decision-making with respect to understanding the potential cardiovascular health impacts of these novel products.

Background

The motivation for the AOP development, a deeper explaination of the underlying biology, and KE/KER assessments can be found in Lowe et al. 2017.

Originally, "oxidative stress" was proposed as the MIE for this AOP. Upon discussion with EAGMST, it was suggested to use a term that is more representative of the mechanism of action, hence "peptide oxidation" is proposed instead. All references to "oxidative stress" within this wiki are made within the context that localised conditions conducive to oxidative damage within the vascular endothelium would trigger the revised MIE, and are limited to the peptide targets named within the AOP; namely GSH, GTPCH1, AKT and eNOS (although other redox sensitive peptides are undoubtedly affected also).

The mechanisms of potentially deleterious peptide oxidation by ROS/RNS are discussed by Berlett and Stadtman (1997) in the context of health effects. References to "oxidative stress" within this wiki are made with this context in mind.

Summary of the AOP

Stressors

Name	Evidence
Reactive oxygen species	Strong

Reactive oxygen species

Compounds or environmental conditions, which generate endothelium-localised ROS *in vivo* are the primary source of the MIE. Notable examples include:

ROS/ROS donors : (Song et al. 2008, van Gorp et al. 1999, van Gorp et al. 2002, Park et al. 2013, Montecinos et al. 2007, Schuppe et al. 1992,

Hypoxia/ischaemia : Nozik-Grayck et al. 2014, Zhang et al. 2014, De Pascali et al. 2014,

SIN-1 (CAS № 16142-27-1) : Das et al. 2014

Heavy Metals (Lead, Cadmium, Mercury) : Vaziri et al. 2001, Wolf et al. 2007

Carbonyls (including methylglyoxal, *N,N*-bis(2-chloroethyl)-*N*-nitroso-urea), acrolein) : Morgan et al. 2014, Dhar et al. 2010, Su et al. 2013, Chen et al. 2010, Chen et al. 2011, Michaud et al. 2006, Qin et al. 2016, Zhang et al. 2011

Glucose : Zou et al. 2002, Song et al. 2007, Du et al. 2013, Du et al. 2001, Dhar et al. 2010, Su et al. 2008

Ultra-fine particulates : Du et al. 2013, Tseng et al. 2016

Cigarette smoke (known to contain carbonyls, metals and ROS) : Michaud et al. 2006, Zhang et al. 2006, Talukder et al. 2011

Molecular Initiating Event

Title	Short name
Peptide Oxidation (https://aopwiki.org/events/209)	Peptide Oxidation

209: Peptide Oxidation (https://aopwiki.org/events/209)

Short Name: Peptide Oxidation

Key Event Component

Process	Object	Action
oxidative stress		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
27: Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11) (https://aopwiki.org/aops/27)	KeyEvent
108: Inhibition of pyruvate dehydrogenase kinase leading to hepatocellular adenomas and carcinomas (in mouse and rat) (https://aopwiki.org/aops/108)	KeyEvent
144: Lysosomal damage leading to liver inflammation (https://aopwiki.org/aops/144)	KeyEvent
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	MolecularInitiatingEvent
210: Activation of c-Jun N-terminal kinase (JNK) and Forkhead box O (FOXO) and reduction of WNT pathways leading to reproductive failure: Integrated multi-OMICS approach for AOP building (https://aopwiki.org/aops/210)	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rodents	rodents	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
human and other cells in culture	human and other cells in culture	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The concentrations of GSH and GSSG have been shown in tissues of human and laboratory animals, including rats, mice and cows (Chen et al., 2010; Giustarini et al., 2013).

How this Key Event Works

Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation. The term oxidative stress indicates the outcome of oxidative damage to biologically relevant macromolecules such as nucleic acids, proteins, lipids and carbohydrates. This occurs when oxidative stress-related molecules, generated in the extracellular environment or within the cell, exceed cellular antioxidant defenses. Major reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and superoxide anion, as well as 4-hydroxy- 2,3-nonenal (HNE) and related 4-hydroxy-2,3-alkenals (HAKs), major aldehydic end-products of lipid peroxidation, can act as potential mediators able to affect signal transduction pathways as well as the proliferative and functional response of target cells. H2O2 and superoxide anion may be also generated as molecular messengers within the cell as part of the cellular response to defined growth factors, cytokines and other mediators. The final consequence at tissue, cellular and molecular level is primarily affected by the steady state concentration of oxidative stress-related molecules. The main biological targets of free radicals are proteins, lipids and DNA.

Major consequences of reaction of ROS, HAKs and NO with biologically relevant macromolecules that can mediate pathophysiological effects:

ROS: DNA: oxidation, strand breaks, genotoxicity Proteins: oxidation, fragmentation, formation of carbonyls Lipids: lipid peroxidation and degradation

HAKs: DNA: adducts (low doses), strand breaks, genotoxicity (high doses) Proteins: adducts (Michael type reactions on Lys, Cys and His residues)

NO: DNA: oxidation, strand breaks Proteins: oxidation, nitrosation, nitration (nytrosylation of tyrosine) Lipids: lipid peroxidation and degradation

Continued oxidative stress can lead to chronic inflammation. Oxidative stress can activate a variety of transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2. Activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines and chemokines, which can activate inflammatory pathways. [1] [2] [3]

Glutathione (GSH) oxidation refers to the conversion of reduced glutathione to its oxidized form glutathione disulfide (GSSG) in the presence of oxidative species. GSH plays an important role as an anti-oxidant in regulating cellular redox homeostasis, and is mainly present in the cell as the reduced form (98%). Deficiency in GSH or a decrease in GSH/GSSG ratio results in decreased anti-oxidant function and increased susceptibility to oxidative stress, thus making it a marker of cellular redox status. An imbalance in GSH/GSSG ratio has been implicated in the onset and progression of human diseases, such as neurodegenerative diseases, cancers, pulmonary diseases and cardiovascular diseases (Ballatori et al., 2009; Kalinina et al., 2014)

How it is Measured or Detected

measuring oxidative stress

*Agents for **ROS detection** are primarily fluorescence based, but recently luminescent based detections have been introduced. The biggest difficulty reported with much of the cellular ROS research has been with the lack of reporter agents specific for discrete molecules. ROS moieties by their nature are reactive with a number of different molecules; as such designing reporter agents has been difficult. With more specific chemistries, particularly for hydrogen peroxide, the specific mechanisms for regulation will be elucidated.*

***Reduced glutathione** (GSH) is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase GSSH + NADPH + H+ à 2 GSH + NADP+ Due to the rapid nature of the reduction of GSSH relative to its synthesis or secretion, the ratio of GSH to GSSH is a good indicator of oxidative stress within cells. GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically in microplates. Several different assays have been designed to measure glutathione in samples. By using a luciferin derivative in conjunction with glutathione S-transferase enzyme the amount of GSH would be proportional to the luminescent signal generated when luciferase is added in a subsequent step. Total glutathione can be determined colorimetrically by reacting GSH with DTNB (Ellman's reagent) in the presence of glutathione reductase. Glutathione reductase reduces GSSH to GSH, which then reacts with DTNB to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 412 nm.*

***Lipid peroxidation** is one of the most widely used indicators of free radical formation, a key indicator of oxidative stress. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA) reactive compounds such as malondialdehyde generated from the decomposition of lipid peroxidation products. While this method is controversial in that it is quite sensitive, but not necessarily specific to MDA, it remains the most widely used means to determine lipid peroxidation. This reaction, which takes place under acidic conditions at 90-100ºC, results in an adduct that can be measured colorimetrically at 532 nm or by fluorescence using a 530 nm excitation wavelength and a 550 nm emission wavelength. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be specific for lipid peroxidation. Unlike the TBA assay, measurement of IsoP appears to be specific to lipid peroxides, they are stable and are not produced by any enzymatic pathway making interpretation easier. There have been a number of commercial ELISA kits developed for IsoPs, but interfering agents in samples requires partial purification of samples prior to running the assay. The only reliable means for detection is through the use of GC/MS, which makes it expensive and limits throughput.*

***Superoxide detection** is based on the interaction of superoxide with some other compound to create a measurable result. The reduction of ferricytochrome c to ferrocytochrome c has been used in a number of situations to assess the rate of superoxide formation. While not completely specific for superoxide this reaction can be monitored colorimetrically at 550 nm. Chemiluminescent reactions have been used for their potential increase in sensitivity over absorbance-based detection methods. The most widely used chemiluminescent substrate is Lucigenin, but this compound has a propensity for redox cycling, which has raised doubts about its use in determining quantitative rates of superoxide production. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical. These dyes are synthesized by reducing the iminium cation of the cyanine (Cy) dyes with sodium borohydride. While weakly fluorescent, upon oxidation their fluorescence intensity increases 100 fold. In addition to being fluorescent, oxidation also converts the molecule from being membrane permeable to an ionic impermeable moiety. The most characterized of these probes are Hydro-Cy3 and Hydro-Cy5.*

***Hydrogen peroxide** (H2O2) is the most important ROS in regards to mitogenic stimulation or cell cycle regulation. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products. The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H2O2 form increasing amounts of fluorescent product.*

***Nitric Oxide** The free radical nitric oxide (•NO) is produced by a number of different cell types with a variety of biological functions. Regardless of the source or role, the free radical •NO has a very short half life (t½= 4 seconds), reacting with several different molecules normally present to form either nitrate (NO3-) or nitrite (NO2-) A commonly used method for the indirect determination of •NO is the determination of its composition products nitrate and nitrite colorimetrically. This reaction requires that nitrate (NO3) first be reduced to nitrite (NO2), typically by the action of nitrate reductase. Subsequent determination of nitrite by a two step process provides information on the "total" of nitrate and nitrite. In the presence of hydrogen ions nitrite forms nitrous acid, which reacts with sulfanilamide to produce a diazonium ion. This then coupled to N-(1-napthyl) ethylenediamine to form the chromophore which absorbs at 543 nm. Nitrite only determinations can then be made in a parallel assay where the samples were not reduced prior to the colorimetric assay. Actual nitrate levels are then calculated by the subtraction of nitrite levels from the total. [4]*

References

Ballatori, N., Krance, S.M., Notenboom, S., Shi, S., Tieu, K., and Hammond, C.L. (2009). Glutathione dysregulation and the etiology and progression of human diseases. Biol. Chem. 390, 191–214.

Chen, C.-A., Wang, T.-Y., Varadharaj, S., Reyes, L.A., Hemann, C., Talukder, M.A.H., Chen, Y.-R., Druhan, L.J., and Zweier, J.L. (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 468, 1115–1118.

Giustarini, D., Dalle-Donne, I., Milzani, A., Fanti, P., and Rossi, R. (2013). Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. Nat. Protoc. 8, 1660–1669.

Held P., 2010 Biotek, Measurement of ROS in Cells, http://www.biotek.com/assets/tech_resources/ROS%20Application%20Guide.pdf (http://www.biotek.com/assets/tech_resources/ROS%20Application%20Guide.pdf)

Kalinina, E.V., Chernov, N.N., and Novichkova, M.D. (2014). Role of glutathione, glutathione transferase, and glutaredoxin in regulation of redox-dependent processes. Biochem. Biokhimiia 79, 1562–1583.

Kamencic, H., Lyon, A., Paterson, P.G., and Juurlink, B.H. (2000). Monochlorobimane fluorometric method to measure tissue glutathione. Anal. Biochem. 286, 35–37.

Parola, M. and Robino, G. (2001). Oxidative stress-related molecules and liver fibrosis. J Hepatol. 35, 297-306

Reuter S. et al., (2010) Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med.49, 1603-1616

Sánchez-Valle V. et al., (2012) Role of oxidative stress and molecular changes in liver fibrosis: a review. Curr Med Chem. 19, 4850-4860

Tipple, T.E., and Rogers, L.K. (2012). Methods for the Determination of Plasma or Tissue Glutathione Levels. Methods Mol. Biol. Clifton NJ 889, 315–324

Key Events

Title	Short name
KE1 : S-Glutathionylation, eNOS (https://aopwiki.org/events/927)	S-Glutathionylation, eNOS
KE2 : Decrease, GTPCH-1 (https://aopwiki.org/events/935)	Decrease, GTPCH-1
KE3 : Decrease, Tetrahydrobiopterin (https://aopwiki.org/events/934)	Decrease, Tetrahydrobiopterin
KE4 : Uncoupling, eNOS (https://aopwiki.org/events/932)	Uncoupling, eNOS
KE6 : Depletion, Nitric Oxide (https://aopwiki.org/events/933)	Depletion, Nitric Oxide
KE7 : Impaired, Vasodilation (https://aopwiki.org/events/937)	Impaired, Vasodilation
KE8 : Increase, Vascular Resistance (https://aopwiki.org/events/951)	Increase, Vascular Resistance
KE5 : Decrease, AKT/eNOS activity (https://aopwiki.org/events/973)	Decrease, AKT/eNOS activity

927: KE1 : S-Glutathionylation, eNOS (https://aopwiki.org/events/927)

Short Name: S-Glutathionylation, eNOS

Key Event Component

Process	Object	Action
protein glutathionylation	nitric oxide synthase, endothelial	increased
protein glutathionylation	cysteine residue	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Organization

Level of Biological Organization
Molecular

Cell term

Cell term
endothelial cell of vascular tree

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

S-glutathionylation of eNOS has been demonstrated in humans, cows, mice and rats (Chen et al., 2010; De Pascali et al., 2014; Du et al., 2013).

How this Key Event Works

S-glutathionylation is a redox-dependent, reversible post-translational modification that is involved in the regulation of various regulatory, structural, and metabolic proteins (Pastore and Piemonte, 2013). Under oxidative stress, S-glutathionylation targets cysteine residues of a protein and adds glutathione through thiol-disulfide exchange with oxidized glutathione (GSSG) or reaction of oxidant-induced protein thiyl radicals with reduced glutathione (Chen et al., 2010, 2011, Schuppe et al. 1992). Endothelial nitric oxide synthase (eNOS) regulates vascular function by generating nitric oxide which is involved in endothelium-dependent relaxation, and control of blood pressure and vascular tone. It has been shown that cysteine residues are important for the maintenance of normal eNOS function. Under oxidative stress, S-glutathionylation of eNOS was induced by GSSG at residue sites Cys 689 and Cys 908, resulting in a decrease in eNOS activity and an increase in superoxide generation, also known as eNOS uncoupling. Furthermore, eNOS S-glutathionylation was shown to be abundant in the vessel walls of spontaneously hypertension rats (SHRs), in contrast to non-hypertensive rats. SHRs demonstrated impaired endothelium-dependent vasodilation, which was reversible upon administration of the reducing agent, dithiothreitol (Chen et al. 2010). Similarly in human aortic endothial cells, exposure to ultrafine particles caused a decrease in NO production in a dose-depedent manner. This was shown to be prevented upon over-expression of glutaredoxin-1, which inhibits eNOS S-glutathionylation (Du et al. 2013).

How it is Measured or Detected

There are four general approaches to detect protein S-glutathionylation (Pastore and Piemonte, 2013).

1. **Quantification of Total S-Glutathionylated Proteins**: Use sample lysis or homogenization in non-reducing buffer containing N-ethylmaleimide to eliminate thiols, followed by protein precipitation, reduction of gluthionyl-protein adducts, and derivatization of protein thiols or free glutathione with fluorescence probes. Fluorescence can be measured by fluorometric analysis with or without prior HPLC separation. This method allows for quantification of glutathionylated proteins but cannot detect glutathione adducts on specific proteins.
2. **Labeling of Glutathione**: Use ^{35}S-cysteine radiolabeling or biotin labeling to detect glutathione adducts on S-thiolated proteins.
3. **Use of Anti-Glutathione Antibodies**: Use commercially available anti-glutathione to detect glutathionylated proteins by Western blots, immunoprecipitation or immunocytolocalization. This method is useful for analysis of individual proteins like eNOS but not for large-scale detection of glutathionylated proteins.
4. **Top-Down Proteomic Approach**: Use liquid chromatography-coupled mass spectrometry to identify S-glutathionylated proteins on whole protein extract from cells without using labeling or anti-glutathione antibody.

References

Chen, C.-A., Wang, T.-Y., Varadharaj, S., Reyes, L.A., Hemann, C., Talukder, M.A.H., Chen, Y.-R., Druhan, L.J., and Zweier, J.L. (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 468, 1115–1118.

Chen CA, Lin CH, Druhan LJ, Wang TY, Chen YR, Zweier JL. Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. J Biol Chem. 2011 286(33):29098-107.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Du, Y., Navab, M., Shen, M., Hill, J., Pakbin, P., Sioutas, C., Hsiai, T.K., and Li, R. (2013). Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. 436, 462–466.

Pastore, A., and Piemonte, F. (2013). Protein glutathionylation in cardiovascular diseases. Int. J. Mol. Sci. 14, 20845–20876.

Schuppe I, Moldéus P, and Cotgreave IA. Protein-specific S-thiolation in human endothelial cells during oxidative stress. (1992) Biochem. Pharmacol. 44: 1757–1764.

935: KE2 : Decrease, GTPCH-1 (https://aopwiki.org/events/935)

Short Name: Decrease, GTPCH-1

Key Event Component

Process	Object	Action
proteasome complex disassembly	GTP cyclohydrolase 1	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell of vascular tree

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Several studies showed decreased GTPCH-1 activity and/or protein expression in cardiac reperfusion patients, bovine endothelial cells, a mouse model of diabetes and a rat model of hypertension (Cervantes-Pérez et al., 2012; Abdelghany et al., 2017; Jayaram et al., 2015; Zhao et al., 2013).

Furthermore, mice deficient in GTPCH-1 demonstrate decreased BH4 bioavailability, increased eNOS uncoupling, pulmonary vascular resistance and pulmonary hypertension (Belik et al. 2011, Nandi et al. 2005, Khoo et al. 2005).

How this Key Event Works

Guanosine triphosphate cyclohydrolase-1 (GTPCH-1) is the rate-limiting enzyme in the de novo biosynthesis of tetrahydrobiopterin (BH4), an essential cofactor for endothelial nitric oxide synthase (eNOS) and nitric oxide generation (Wang et al., 2008). GTPCH-1 catalyzes the rearrangement of GTP to 7-dihydroneopterin triphosphate, which is converted to BH4 through sequential actions of pyruvoyl tetrahydrobiopterin synthase and sepiapterin reductase. GTPCH-1 activity is regulated in a negative feedback by levels of BH4 which promotes binding of GTPCH-1 with its inhibitor GTPCH feedback regulatory protein (GFRP), but phosphorylation of GTPCH-1 reduces its binding to GFRP and prevents this negative feedback (Chen et al., 2011). Loss or inactivation of GTPCH-1 results in decreased BH4 levels, which causes eNOS uncoupling.

How it is Measured or Detected

The activity of GTPCH-1 can be detected through the quantification of neopterin by high-performance liquid chromatography (HPLC) after the conversion of enzymatically formed dihydroneopterin triphosphate into neopterin by sequential iodine oxidation and dephosphorylation.

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review).

Belik J, McIntyre BA, Enomoto M, Pan J, Grasemann H, Vasquez-Vivar J. Pulmonary hypertension in the newborn GTP cyclohydrolase I-deficient mouse. Free Radic Biol Med. 2011, 51(12):2227-33.

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Chen, W., Li, L., Brod, T., Saeed, O., Thabet, S., Jansen, T., Dikalov, S., Weyand, C., Goronzy, J., and Harrison, D.G. (2011). Role of increased guanosine triphosphate cyclohydrolase-1 expression and tetrahydrobiopterin levels upon T cell activation. J. Biol. Chem. 286, 13846–13851.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Khoo JP, Zhao L, Alp NJ, Bendall JK, Nicoli T, Rockett K, et al. Pivotal role for endothelial tetrahydrobiopterin in pulmonary hypertension. Circulation. 2005;111:2126–33

Nandi M, Miller A, Stidwill R, Jacques TS, Lam AA, Haworth S, et al. Pulmonary hypertension in a GTP-cyclohydrolase 1-deficient mouse. Circulation. 2005;111:2086–90

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Zhao, Y., Wu, J., Zhu, H., Song, P., and Zou, M.-H. (2013). Peroxynitrite-dependent zinc release and inactivation of guanosine 5'-triphosphate cyclohydrolase 1 instigate its ubiquitination in diabetes. Diabetes 62, 4247–4256.

934: KE3 : Decrease, Tetrahydrobiopterin (https://aopwiki.org/events/934)

Short Name: Decrease, Tetrahydrobiopterin

Key Event Component

Process	Object	Action
biosynthetic process	5,6,7,8-tetrahydrobiopterin	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell of vascular tree

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Decreased BH4 is observed in humans (Jayaram et al., 2015), cows (Abdelghany et al., 2017; Whitsett et al., 2007, Wang et al., 2008), mice (Adlam et al., 2012; Chuaiphichai et al., 2014; Crabtree et al., 2009; Tatham et al., 2009; Wang et al., 2008) and rats (Cervantes-Pérez et al., 2012).

How this Key Event Works

Tetrahydrobiopterin (BH4) is an essential cofactor for a group of enzymes including aromatic acid hydroxylases, nitric oxide synthase (NOS) isoforms, and alkylglycerol monooxygenase (Wang et al., 2014). BH4 is synthesized from guanosine triphosphate through sequential reactions catalyzed by enzymes GTPCH-1 (https://aopwiki.org/wiki/index.php/Event:935), pyruvoyl tetrahydropterin synthase, and sepiapterin reductase (Tatham et al., 2009). During NOS catalysis, BH4 donates electrons to the ferrous-dioxygen complex in the oxygenase domain, leading to oxidation of L-arginine to N-hydroxy-Larginine and eventually conversion to citrulline and nitric

oxide production (Chen et al., 2011; Crabtree et al., 2009). BH4 also stabilizes dimers of NOS isoforms, which is required for their enzymatic activity. When BH4 levels are decreased or limited, for example under oxidative stress conditions, BH4 can be oxidized to dihydrobiopterin (BH2) and then converted to biopterin. This reduction in BH4 availability results in NOS uncoupling where NOS is uncoupled from L-arginine oxidation and superoxide (or other reactive species) is produced rather than nitric oxide (Carnicer et al., 2012). Decreased BH4 have been demonstrated in a variety of vascular diseases such as hypertension, diabetes and atherosclerosis where endothelial dysfunction occurs.

How it is Measured or Detected

Levels of BH4, BH2 and biopterin levels can be determined by reverse-phase high-performance liquid chromatography (HPLC) followed by electrochemical detection (for BH4) and fluorescence detection (for BH2 and biopterin) (Howells et al., 1986).

A LC-MS/MS method has been published by Zhao et al. (2009), which was validated for detection in human, monkey, dog, rabbit, rat and mouse plasma, and used to support a successful drug approval submission.

ELISA kits for BH4 are also commercially available.

In each case, care must be taken to protect the sample from oxidation, and BH4 is highly redox sensitive. Dithioerythritol is commonly used as a preservation agent.

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review).

Adlam, D., Herring, N., Douglas, G., De Bono, J.P., Li, D., Danson, E.J., Tatham, A., Lu, C.-J., Jennings, K.A., Cragg, S.J., et al. (2012). Regulation of β-adrenergic control of heart rate by GTP-cyclohydrolase 1 (GCH1) and tetrahydrobiopterin. Cardiovasc. Res. 93, 694–701.

Carnicer, R., Hale, A.B., Suffredini, S., Liu, X., Reilly, S., Zhang, M.H., Surdo, N.C., Bendall, J.K., Crabtree, M.J., Lim, G.B.S., et al. (2012). Cardiomyocyte GTP cyclohydrolase 1 and tetrahydrobiopterin increase NOS1 activity and accelerate myocardial relaxation. Circ. Res. 111, 718–727.

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Chen, W., Li, L., Brod, T., Saeed, O., Thabet, S., Jansen, T., Dikalov, S., Weyand, C., Goronzy, J., and Harrison, D.G. (2011). Role of increased guanosine triphosphate cyclohydrolase-1 expression and tetrahydrobiopterin levels upon T cell activation. J. Biol. Chem. 286, 13846–13851.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

Chuaiphichai, S., McNeill, E., Douglas, G., Crabtree, M.J., Bendall, J.K., Hale, A.B., Alp, N.J., and Channon, K.M. (2014). Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 64, 530–540.

Howells, D.W., Smith, I., and Hyland, K. (1986). Estimation of tetrahydrobiopterin and other pterins in cerebrospinal fluid using reversed-phase high-performance liquid chromatography with electrochemical and fluorescence detection. J. Chromatogr. 381, 285–294.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Tatham, A.L., Crabtree, M.J., Warrick, N., Cai, S., Alp, N.J., and Channon, K.M. (2009). GTP cyclohydrolase I expression, protein, and activity determine intracellular tetrahydrobiopterin levels, independent of GTP cyclohydrolase feedback regulatory protein expression. J. Biol. Chem. 284, 13660–13668.

Wang, Q., Yang, M., Xu, H., and Yu, J. (2014). Tetrahydrobiopterin improves endothelial function in cardiovascular disease: a systematic review. Evid.-Based Complement. Altern. Med. ECAM 2014, 850312.

Whitsett, J., Picklo, M.J., and Vasquez-Vivar, J. (2007). 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation. Arterioscler. Thromb. Vasc. Biol. 27, 2340–2347.

Zhao Y, Cao J, Chen YS, Zhu Y, Patrick C, Chien B, Cheng A, Foehr ED. Detection of tetrahydrobiopterin by LC-MS/MS in plasma from multiple species. Bioanalysis. 2009;1(5):895-903.

932: KE4 : Uncoupling, eNOS (https://aopwiki.org/events/932)

Short Name: Uncoupling, eNOS

Key Event Component

Process	Object	Action
	nitric oxide synthase, endothelial	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

eNOS uncoupling has been demonstrated in humans, cows, mice and rats (Chen et al., 2010; Crabtree et al., 2009; De Pascali et al., 2014; Du et al., 2013; Jayaram et al., 2015).

How this Key Event Works

Endothelial nitric oxide synthase (eNOS) is responsible for the generation of vascular nitric oxide (NO), a protective molecule that is involved in the regulation of endothelium-dependent vasodilation, vascular tone, and blood pressure (Förstermann and Münzel, 2006). To generate NO, eNOS hydroxylates L-arginine to N-hydroxy-L-arginine and then oxidizes N-hydroxy-L-arginine to L-citrulline and NO. This enzymatic process requires NADPH, Ca2+/calmondulin, flavin mononucleotide, flavin adenine dinucleotide and its cofactor tetrahydrobiopterin (BH4) (https://aopwiki.org/wiki/index.php/Event:934). Limiting BH4 levels or S-glutathionylation of eNOS (https://aopwiki.org/wiki/index.php/Event:927) can lead to eNOS uncoupling in which eNOS produces superoxide (or other reactive oxygen species) and less NO. The uncoupling of eNOS has been demonstrated to cause endothelial dysfunction, and is implicated in a number of cardiovascular diseases such as hypertension, atherosclerosis, hypercholesterolemia, and diabetes mellitus (Dumitrescu et al., 2007).

How it is Measured or Detected

The activity of eNOS can be measured indirectly through superoxide and NO production. Superoxides can be detected using several standard methods including lucigenin-enhanced chemiluminescence (Münzel et al., 2002; Tarpey et al., 1999), electron paramagnetic resonance (EPR) spin-trapping (Roubaud et al., 1997), and HPLC/fluorescence detector-based assay using dihydroethidium (Fink et al., 2004; Zhao et al., 2003). NO production can be measured through the conversion of L-arginine to L-citrulline (de Bono et al., 2007) , in situ fluorescent signal detection with fluorescent indicator DAF-2 DA (Itoh et al., 2000; Nagata et al., 1999; Qiu et al., 2001), EPR spin-trapping (Xia et al., 2000), and the determination of total nitrate and nitrite concentration (Crabtree et al., 2009; Du et al., 2013).

References

de Bono, J.P., Warrick, N., Bendall, J.K., Channon, K.M., and Alp, N.J. (2007). Radiochemical HPLC detection of arginine metabolism: Measurement of nitric oxide synthesis and arginase activity in vascular tissue. Nitric Oxide 16, 1–9.

Chen, X., Xu, J., Feng, Z., Fan, M., Han, J., and Yang, Z. (2010). Simvastatin combined with nifedipine enhances endothelial cell protection by inhibiting ROS generation and activating Akt phosphorylation. Acta Pharmacol. Sin. 31, 813–820.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Du, Y., Navab, M., Shen, M., Hill, J., Pakbin, P., Sioutas, C., Hsiai, T.K., and Li, R. (2013). Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. 436, 462–466.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Fink, B., Laude, K., McCann, L., Doughan, A., Harrison, D.G., and Dikalov, S. (2004). Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. Am. J. Physiol. Cell Physiol. 287, C895–C902.

Förstermann, U., and Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.

Itoh, Y., Ma, F.H., Hoshi, H., Oka, M., Noda, K., Ukai, Y., Kojima, H., Nagano, T., and Toda, N. (2000). Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. Anal. Biochem. 287, 203–209.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Münzel, T., Afanas'ev, I.B., Kleschyov, A.L., and Harrison, D.G. (2002). Detection of Superoxide in Vascular Tissue. Arterioscler. Thromb. Vasc. Biol. 22, 1761–1768.

Nagata, N., Momose, K., and Ishida, Y. (1999). Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. J. Biochem. (Tokyo) 125, 658–661.

Qiu, W., Kass, D.A., Hu, Q., and Ziegelstein, R.C. (2001). Determinants of shear stress-stimulated endothelial nitric oxide production assessed in real-time by 4,5-diaminofluorescein fluorescence. Biochem. Biophys. Res. Commun. 286, 328–335.

Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P., and Zweier, J.L. (1997). Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. Anal. Biochem. 247, 404–411.

Tarpey, M.M., White, C.R., Suarez, E., Richardson, G., Radi, R., and Freeman, B.A. (1999). Chemiluminescent Detection of Oxidants in Vascular Tissue Lucigenin But Not Coelenterazine Enhances Superoxide Formation. Circ. Res. 84, 1203–1211.

Xia, Y., Cardounel, A.J., Vanin, A.F., and Zweier, J.L. (2000). Electron paramagnetic resonance spectroscopy with N-methyl-D-glucamine dithiocarbamate iron complexes distinguishes nitric oxide and nitroxyl anion in a redox-dependent manner: applications in identifying nitrogen monoxide products from nitric oxide synthase. Free Radic. Biol. Med. 29, 793–797.

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vásquez-Vivar, J., and Kalyanaraman, B. (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic. Biol. Med. 34, 1359–1368.

933: KE6 : Depletion, Nitric Oxide (https://aopwiki.org/events/933)

Short Name: Depletion, Nitric Oxide

Key Event Component

Process	Object	Action
nitric oxide biosynthetic process	nitric oxide	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Organization

Level of Biological Organization
Cellular

Organ term

Organ term
blood

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

NO depletion was observed in humans, cows, mice and rats (Chen et al., 2010; Crabtree et al., 2009; De Pascali et al., 2014; Du et al., 2013; Jayaram et al., 2015).

How this Key Event Works

Nitric oxide (NO), constitutively produced by endothelial nitric oxide synthase (eNOS), is an important regulator of vascular homeostasis. Endothelial-derived NO promotes vasodilation and protects against atherogenesis through the inhibition of vascular smooth muscle cell proliferation and migration, platelet aggregation and adhesion, and leukocyte adherence. Its effects have an influence on vascular resistance, blood pressure, vascular remodeling and angiogenesis (Luo et al., 2000). Dysfunctional eNOS as a result of eNOS uncoupling (https://aopwiki.org/wiki/index.php/Event:932) leads to a decrease or loss of NO bioavailability and an elevation of superoxide production (Crabtree et al., 2009). The imbalance of NO and superoxide is associated with many disorders, such as hypertension, atherosclerosis, hypercholesterolemia, and diabetes mellitus.

How it is Measured or Detected

NO production can be measured through the conversion of L-arginine to L-citrulline (de Bono et al., 2007) , in situ fluorescent signal detection with fluorescent indicator DAF-2 DA (Itoh et al., 2000; Nagata et al., 1999; Qiu et al., 2001), EPR spin-trapping (Xia et al., 2000), and the determination of total nitrate and nitrite concentration (Crabtree et al., 2009; Du et al., 2013).

References

de Bono, J.P., Warrick, N., Bendall, J.K., Channon, K.M., and Alp, N.J. (2007). Radiochemical HPLC detection of arginine metabolism: Measurement of nitric oxide synthesis and arginase activity in vascular tissue. Nitric Oxide 16, 1–9.

Chen, X., Xu, J., Feng, Z., Fan, M., Han, J., and Yang, Z. (2010). Simvastatin combined with nifedipine enhances endothelial cell protection by inhibiting ROS generation and activating Akt phosphorylation. Acta Pharmacol. Sin. 31, 813–820.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Du, Y., Navab, M., Shen, M., Hill, J., Pakbin, P., Sioutas, C., Hsiai, T.K., and Li, R. (2013). Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. 436, 462–466.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Itoh, Y., Ma, F.H., Hoshi, H., Oka, M., Noda, K., Ukai, Y., Kojima, H., Nagano, T., and Toda, N. (2000). Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. Anal. Biochem. 287, 203–209.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Luo, Z., Fujio, Y., Kureishi, Y., Rudic, R.D., Daumerie, G., Fulton, D., Sessa, W.C., and Walsh, K. (2000). Acute modulation of endothelial Akt/PKB activity alters nitric oxide–dependent vasomotor activity in vivo. J. Clin. Invest. 106, 493–499.

Nagata, N., Momose, K., and Ishida, Y. (1999). Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. J. Biochem. (Tokyo) 125, 658–661.

Qiu, W., Kass, D.A., Hu, Q., and Ziegelstein, R.C. (2001). Determinants of shear stress-stimulated endothelial nitric oxide production assessed in real-time by 4,5-diaminofluorescein fluorescence. Biochem. Biophys. Res. Commun. 286, 328–335.

Xia, Y., Cardounel, A.J., Vanin, A.F., and Zweier, J.L. (2000). Electron paramagnetic resonance spectroscopy with N-methyl-D-glucamine dithiocarbamate iron complexes distinguishes nitric oxide and nitroxyl anion in a redox-dependent manner: applications in identifying nitrogen monoxide products from nitric oxide synthase. Free Radic. Biol. Med. 29, 793–797.

937: KE7 : Impaired, Vasodilation (https://aopwiki.org/events/937)

Short Name: Impaired, Vasodilation

Key Event Component

Process	Object	Action
vasodilation	blood vessel	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Organ term

Organ term
circulatory system

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Oryctolagus cuniculus	Oryctolagus cuniculus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9986)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

Vasodilation has been observed in humans, rabbits, mice and rats.

How this Key Event Works

Vasodilation refers to the widening or increase in the diameter of blood vessels (e.g. large arteries, large veins, small arterioles) that is caused by the relaxation of vascular smooth muscle cells (VSMCs) within the walls of blood vessels, thus increasing blood flow and decreasing arterial blood pressure and heart rate (Siddiqui, 2011). VSMC relaxation is regulated through a number of mechanisms, including cyclic GMP-dependent hyperpolarization and relaxation via nitric oxide (NO), cAMP-dependent hyperpolarization via prostaglandins, and stimulation of potassium channels via endothelial-derived hyperpolarizing factors (Durand and Gutterman, 2013). Under oxidative stress, decreased NO bioavailability results in impaired vasodilation, which is associated with cardiovascular diseases such as hypertension (Silva et al., 2012).

How it is Measured or Detected

Endothelium-dependent vasodilation can be measured using invasive and non-invasive methods (Raitakari and Celermajer, 2000). For the invasive approach, vasodilation is measured after intra-arterial pharmacologic stimulation with substances that enhance NO release (e.g. acetylcholine, bradykinin). The non-invasive ultrasound-based method evaluates flow-mediated vasodilation (FMD) in the superficial arteries, such as brachial, radial, or femoral vessels.

Guidelines for the measurement of FMD have been published (Corretti et al. 2002).

References

Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery

Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery. Journal of the American College of Cardiology, 2002, 39 (2) 257-265

Durand, M.J., and Gutterman, D.D. (2013). Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. Microcirc. N. Y. N 1994 20, 239–247.

Raitakari, O.T., and Celermajer, D.S. (2000). Flow-mediated dilatation. Br. J. Clin. Pharmacol. 50, 397–404.

Siddiqui, A. (2011). Effects of Vasodilation and Arterial Resistance on Cardiac Output. J. Clin. Exp. Cardiol. 02.

Silva, B.R., Pernomian, L., and Bendhack, L.M. (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. Front. Physiol. 3, 441.

951: KE8 : Increase, Vascular Resistance (https://aopwiki.org/events/951)

Short Name: Increase, Vascular Resistance

Key Event Component

Process	Object	Action
increased systemic vascular resistance		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Biological Organization

Level of Biological Organization

Level of Biological Organization
Organ

Organ term

Organ term
circulatory system

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

Increased SVR was observed in humans, pigs and rats (Siddiqui 2011, Dikalova et al. 2016,

How this Key Event Works

Vascular resistance is the resistance to blood flow in the circulatory system (Siddiqui, 2011). Systemic vascular resistance (SVR), also known as total peripheral resistance, refers specifically to the resistance to blood flow offered by the peripheral circulation. Vasodilation decreases SVR while vasoconstriction or impaired vasodilation (https://aopwiki.org/wiki/index.php/Event:937) increases SVR.

How it is Measured or Detected

Vascular resistance cannot be measured by any direct means, but can be calculated using a formula: (mean arterial pressure minus mean right arterial pressure) divided by cardiac output (Siddiqui 2011).

References

Siddiqui, A. (2011). Effects of Vasodilation and Arterial Resistance on Cardiac Output. J. Clin. Exp. Cardiol. 02.

Dikalova A, Aschner JL, Kaplowitz MR, Summar M, Fike CD. Tetrahydrobiopterin oral therapy recouples eNOS and ameliorates chronic hypoxia-induced pulmonary hypertension in newborn pigs. Am J Physiol Lung Cell Mol Physiol. 2016, 1;311(4):L743-L753.

973: KE5 : Decrease, AKT/eNOS activity (https://aopwiki.org/events/973)

Short Name: Decrease, AKT/eNOS activity

Key Event Component

Process	Object	Action
catalytic activity	nitric oxide synthase, endothelial	decreased
catalytic activity	AKT kinase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell of vascular tree

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Decreased Akt and eNOS activity was observed in humans, cows, mice and rats following exposure to stressors.

Cigarette smoke exposure was shown to inhibit the phosphorylation of AKT and eNOS in VEGF-stimulated human umbilical vein endothelial cells (HUVECs), resulting in decreased NO levels (Michaud et al. 2006).

In rat aortic rings, exposure to methylglyoxal and high concentrations of glucose decreased endothelium-dependent relaxation. Further experiments in rat endothelial cells and HUVECs demonstrated a reduction in eNOS phosphorylation and activity, and reduced NO levels in response to the same stressors (Dhar et al. 2010).

In bovine aortic endothelial cells, AKT and eNOS phosphorylation were decreased following exposure to the peroxynitrite source; SIN-1, with an associated reduction in NO bioavailability. These effects were ameliorated by treatment with the ROS scavenger DMPO (Das et al. 2014).

eNOS knockout mice are routinely used as models of hypertension. Such mice display reduced bioavailability of NO and impaired vasodilation (Huang et al. 1995).

Reduced AKT/eNOS phosphorylation was reported under conditions of hyperglycaemia (in mice) and in HUVECs following treatment with high concentrations of glucose. Aortic rings from hyperglycaemic mice demonstrated impaired vasodilation. Resveratrol treatment was shown to improve vasodilation and eNOS phosphorylation in wild-type mice, but not AKT knockout mice. Transfection of HUVECs with AKT siRNA abolished resveratrol-enhanced eNOS phosphorylation and NO release (Li et al. 2017),

How this Key Event Works

Endothelial nitric oxide synthase (eNOS) is responsible for the generation of nitric oxide (NO), which is an important regulator of vascular homeostasis. The activity of eNOS can be regulated through calmodulin-mediated dimerization, tetrahydrobiopterin-mediated conversion of L-arginine to L-citrulline, protein-protein interactions with heat shock protein 90 and caveolin, S-nitrosylation, acetylation and phosphorylation (Atochin et al., 2007; Qian and Fulton, 2013). eNOS has been shown to be phosphorylated at multiple sites including tyrosine (Y), serine (Ser) and threonine (Thr) residues. Serine-threonine protein kinase AKT, a multifunctional regulator of cellular processes like glucose metabolism and proliferation, can directly phosphorylate eNOS at Ser1177/Ser1179, leading to increased eNOS enzymatic activity and subsequent NO production (Dimmeler et al., 1999; Fulton et al., 1999). Inhibition of AKT or a mutation of the AKT phosphorylation site on eNOS attenuates eNOS phosphorylation and its activity, resulting in decreased NO bioavailability and endothelial dysfunction (Dimmler et al. 1999)

How it is Measured or Detected

Western blot analysis can be performed to determine the expression levels of phosphorylated eNOS, phosphorylated Akt, total Akt and total eNOS proteins using the appropriate anti-phospho-eNOS, anti-phospho-Akt, anti-eNOS, and anti-Akt antibodies. Alternatively, eNOS activity can be measured using the conversion of L-arginine to L-citrulline assay.

ELISA kits for AKT/eNOS and phospho AKT/eNOS expression are commercially available.

References

Atochin, D.N., Wang, A., Liu, V.W.T., Critchlow, J.D., Dantas, A.P.V., Looft-Wilson, R., Murata, T., Salomone, S., Shin, H.K., Ayata, C., et al. (2007). The phosphorylation state of eNOS modulates vascular reactivity and outcome of cerebral ischemia in vivo. J. Clin. Invest. 117, 1961–1967.

Das A, Gopalakrishnan B, Druhan LJ, Wang TY, De Pascali F, Rockenbauer A, Racoma I, Varadharaj S, Zweier JL, Cardounel AJ, Villamena FA. Reversal of SIN-1-induced eNOS dysfunction by the spin trap, DMPO, in bovine aortic endothelialcells via eNOS phosphorylation. Br J Pharmacol. 2014, 171(9):2321-34. doi: 10.1111/bph.12572.

Dhar A, Dhar I, Desai KM, Wu L. Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. Br J Pharmacol. 2010, 161(8):1843-56.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., and Sessa, W.C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase AKT. Nature 399, 597–601.

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature. 1995, 377(6546):239-42.

Li JY, Huang WQ, Tu RH, Zhong GQ, Luo BB, He Y. Resveratrol rescues hyperglycemia-induced endothelial dysfunction via activation of Akt. Acta Pharmacol Sin. 2017, 38(2):182-191.

Michaud SE, Dussault S, Groleau J, Haddad P, Rivard A.J. Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species. Mol Cell Cardiol. 2006 Aug;41(2):275-84.

Qian, J., and Fulton, D. (2013). Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Oxid. Physiol. 4, 347.

Adverse Outcomes

Title	Short name
Hypertension (https://aopwiki.org/events/952)	Hypertension

952: Hypertension (https://aopwiki.org/events/952)

Short Name: Hypertension

Key Event Component

Process	Object	Action
hypertension		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
149: Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	AdverseOutcome

Stressors

Name
Reactive oxygen species

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

Animal models including mouse and rat models are routinely used to study hypertension, and have been shown to reflect human physiology relating to hypertension (Leong et al., 2015).

How this Key Event Works

Hypertension is an important cardiovascular risk factor and considered one of the leading causes of cardiovascular morbidity and mortality (Kizhakekuttu and Widlansky, 2010). It is defined as a chronic elevation in blood pressure and is characterized by elevated systemic vascular resistance due to dysregulated vasomotor function and structural remodeling (Lee and Griendling, 2008). Although many genetic and environmental factors contribute to the development to hypertension, oxidative stress appears to be the main pathway involved in its pathogenesis. Excessive reactive oxygen species (ROS) contributes to endothelial nitric oxide synthase (eNOS) uncoupling (https://aopwiki.org/wiki/index.php/Event:932), resulting in increased superoxide production but decreased nitric oxide (https://aopwiki.org/wiki/index.php/Event:933) (NO), a critical regulator of vascular homeostasis (Silva et al., 2012). Depletion of NO leads to impaired endothelium-dependent vasodilation (https://aopwiki.org/wiki/index.php/Event:937), thus promoting endothelial dysfunction, which is a hallmark of hypertension.

How it is Measured or Detected

Arterial blood pressure is commonly measured using a sphygmomanometer, which provides systolic and diastolic blood pressure measurements in millimeters of mercury (mmHg).

Pathological hypertension is characterised according to current guidelines; https://www.nice.org.uk/guidance/cg127/evidence

Stage 1 hypertension : Clinic blood pressure is 140/90 mmHg or higher and subsequent ambulatory blood pressure monitoring (ABPM) daytime average or home blood pressure monitoring (HBPM) average blood pressure is 135/85 mmHg or higher.

Stage 2 hypertension : Clinic blood pressure is 160/100 mmHg or higher and subsequent ABPM daytime average or HBPM average blood pressure is 150/95 mmHg or higher.

Severe hypertension : Clinic systolic blood pressure is 180 mmHg or higher or clinic diastolic blood pressure is 110 mmHg or higher.

References

Durand, M.J., and Gutterman, D.D. (2013). Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. Microcirc. N. Y. N 1994 20, 239–247.

Kizhakekuttu, T.J., and Widlansky, M.E. (2010). Natural antioxidants and hypertension: promise and challenges. Cardiovasc. Ther. 28, e20–e32.

Leong, X.-F., Ng, C.-Y., Jaarin, K., Leong, X.-F., Ng, C.-Y., and Jaarin, K. (2015). Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis, Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis. BioMed Res. Int. BioMed Res. Int. 2015, 2015, e528757.

Silva, B.R., Pernomian, L., and Bendhack, L.M. (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. Front. Physiol. 3, 441.

Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Peptide Oxidation	directly leads to	KE2 : Decrease, GTPCH-1	Moderate	Weak
KE1 : S-Glutathionylation, eNOS	directly leads to	KE4 : Uncoupling, eNOS	Strong	Moderate
KE2 : Decrease, GTPCH-1	directly leads to	KE3 : Decrease, Tetrahydrobiopterin	Strong	Strong
KE3 : Decrease, Tetrahydrobiopterin	directly leads to	KE4 : Uncoupling, eNOS	Strong	Strong
KE4 : Uncoupling, eNOS	directly leads to	KE6 : Depletion, Nitric Oxide	Strong	Strong
KE6 : Depletion, Nitric Oxide	directly leads to	KE7 : Impaired, Vasodilation	Strong	Moderate
KE7 : Impaired, Vasodilation	directly leads to	KE8 : Increase, Vascular Resistance	Moderate	Weak
KE8 : Increase, Vascular Resistance	directly leads to	Hypertension	Moderate	Weak
KE5 : Decrease, AKT/eNOS activity	directly leads to	KE6 : Depletion, Nitric Oxide	Strong	Strong
Peptide Oxidation	directly leads to	KE5 : Decrease, AKT/eNOS activity	Strong	Moderate
Peptide Oxidation	directly leads to	KE1 : S-Glutathionylation, eNOS	Moderate	Weak

Peptide Oxidation leads to Decrease, GTPCH-1 (https://aopwiki.org/relationships/980)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Bos taurus	Bos taurus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

Several studies showed decreased GTPCH-1 activity and/or protein expression under oxidative stress in cardiac reperfusion patients, bovine endothelial cells, a mouse model of diabetes and a rat model of hypertension (Cervantes-Pérez et al., 2012; AbdelGhany et al., (under review); Jayaram et al., 2015; Zhao et al., 2013).

How Does This Key Event Relationship Work

Exposure to known inducers of oxidative stress such as cigarette smoke extract (AbdelGhany et al., under review) or peroxynitrite (Zhao et al., 2013) causes the loss of GTPCH-1 activity, resulting in decreased levels of tetrahydrobiopterin (BH4) and subsequent uncoupling of endothelial nitric oxide synthase (eNOS).

Weight of Evidence

Biological Plausibility
Several studies demonstrated that GTPCH-1 is also affected by oxidative stress, which provides evidence for moderate biological plausibility. In vitro exposure to chemically synthesized peroxynitrite inhibited GTPCH-1 activity in a dose-dependent manner (Zhao et al., 2013). This inhibition as well as increased ubiquitination of GTPCH-1 were observed in cultured bovine aortic endothelial cells (BAECs) and streptozotocin-induced diabetes in mice following peroxynitrite treatment. Ubiquitination of GTPCH-1 leads to its degradation, which is equivalent to a decrease in GTPCH-1. In another study, GTPCH-1 levels were reduced by cigarette smoke extract (CSE) exposure in BAECs (AbdelGhany et al., under review). There is also evidence that CSE promoted GTPCH-1 degradation by increasing proteasomal activity. Furthermore, cardiac reperfusion patients experienced oxidative stress which was associated with reduced GTPCH-1 activity (Jayaram et al., 2015).

Empirical Support for Linkage
Include consideration of temporal concordance here

In a rat model of aortic coarctation-associated hypertension, increased ROS (1.75 nmol DCF/mg protein/min) and decreased GTPCH-1 protein expression (western blot band intensity of 0.0087±0.005) were observed (Cervantes-Pérez et al., 2012). However, following clofibrate treatment (100 mg/kg/day, 7 days), ROS was reduced by approximately 30% and GTPCH-1 band intensity increased to 0.0087±0.005. This study provides evidence that there is a dependent change between oxidative stress and GTPCH-1, but we found no dose-response relationship, so empirical support for this KER is weak.

Uncertainties or Inconsistencies
No uncertainties or inconsistencies were found for this KER.

Quantitative Understanding of the Linkage
Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

As the relationship between oxidative stress and GTPCH-1 is not well-studied, there is limited quantitative understanding of this linkage.

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review)

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49

Zhao, Y., Wu, J., Zhu, H., Song, P., and Zou, M.-H. (2013). Peroxynitrite-dependent zinc release and inactivation of guanosine 5'-triphosphate cyclohydrolase 1 instigate its ubiquitination in diabetes. Diabetes 62, 4247–4256.

S-Glutathionylation, eNOS leads to Uncoupling, eNOS (https://aopwiki.org/relationships/955)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Term	Scientific Term	Evidence	Links
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The evidence supporting this key event relationship are from human subjects, HAECs, BAECs, and SHR rats (Chen et al., 2010; De Pascali et al., 2014; Du et al., 2013; Jayaram et al., 2015).

How Does This Key Event Relationship Work

Oxidative stress can trigger S-glutathionylation of eNOS at cysteine residues Cys689 and Cys908, which are known to be critical for normal eNOS function (Zweier et al., 2011). S-glutathionylation directly causes eNOS uncoupling, a state in which eNOS switches from producing NO to generating superoxide, thus impairing endothelium-dependent vasodilation and contributing to endothelial dysfunction. Uncoupling of eNOS via S-glutathionylation is different from BH4-mediated eNOS uncoupling in that superoxide is produced in the reductase domain rather than the oxygenase domain and superoxide generation cannot be inhibited by L-NG-nitroarginine methyl ester (L-NAME), suggesting that S-glutathionylation occurs independent of calcium/calmodulin and heme.

Weight of Evidence

Biological Plausibility

In vitro experiments showed that S-glutathionylation of eNOS significantly decreased NO activity in dose-dependent manner and greatly increased superoxide generation (Chen et al., 2010). NOS inhibitor L-NAME partially blocked superoxide generation. These results were observed in bovine aortic endothelial cells (BAECs) and in aortae of spontaneously hypertensive (SHR) rats. Treatment of BAECs with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase, induced eNOS S-glutathionylation, resulting in decreased NO and increased superoxide generation. Gene silencing of eNOS in BAECs also induced superoxide production. In aortae of SHR rats, S-glutathionylation as determined by immunohistology was associated with a decrease in endothelium-dependent vasodilation as a result of decreased NO. Exposure of human aortic endothelial cells (HAECs) to ultrafine particles (UFP) caused S-glutathionylation of eNOS and a dose-dependent decrease in NO production (Du et al., 2013). Decreased NO production was found to be mediated by S-glutathionylation since overexpression of glutaredoxin, an inhibitor of S-glutathionylation, significantly reduced UPF-mediated decrease in NO production. Cardiac reperfusion patients exhibited decreased eNOS activity which was identified to be a result of eNOS S-glutathionylation (Jayaram et al., 2015). Additional evidence was observed in BAECs undergoing hypoxia and reoxygenation in which eNOS S-glutathionylation increased by three-fold compared to control cells and NO production was decreased (De Pascali et al., 2014). These effects were reversed with treatment of N-acetyl-l-cysteine, which increased cellular concentration of GSH. These results demonstrate a clear interaction between S-glutathionylation and eNOS uncoupling, therefore this link has strong biological plausibility.

Empirical Support for Linkage

Include consideration of temporal concordance here

Treatment with BCNU (25 µM, 80 µM) resulted in increased eNOS S-glutathionylation, increased superoxide generation and decreased NO production in a dose-dependent manner in BAECs (Chen et al., 2010).

Exposure to hypoxia/reoxygenation and treatment with angiotensin II (Ang II) demonstrated a response-response relationship between eNOS S-glutathionylation and superoxide generation in human and bovine endothelial cells (De Pascali et al., 2014; Galougahi et al., 2014). Following hypoxia and reoxygenation in BAECs, increased S-glutathionylation of eNOS (0.3 to 1 arbitray units; measured by western blot), decreased NO production (100% to 34.2 ± 1.7%) and increased superoxide generation (relative intensity of 3.5 to 49.2; by fluorescence) were observed (De Pascali et al., 2014). Treatment with 100 nmol/L Ang II for 1 hour caused an increase in both eNOS S-glutathionylation (from 1 to 1.42 fold) and superoxide generation (from 81.1 to 113 fluorescence signal unit) (Galougahi et al., 2014).

Overall, there is moderate empirical support for eNOS S-glutathionylation leading to eNOS uncoupling.

Uncertainties or Inconsistencies

No uncertainties or inconsistencies were found for this KER.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

No study explored how much change in S-glutathionylation of eNOS is required to lead to eNOS uncoupling. One study in HUVECs showed treatment with 100nM/L Ang II increased S-glutathionylated eNOS protein expression by 1.42 fold, and was associated with an increase in superoxide generation of nearly 50% at the same dose. DTT (which removes PR-SG residues) was shown to partially reverse this effect (Galougahi et al., 2014). Ang II and hypoxia/reoxygenation were demonstrated to be modulators of the response-response relationship between S-glutathionylation of eNOS and eNOS uncoupling as these two stressors caused a change in both key events (De Pascali et al., 2014; Galougahi et al., 2014). More experiments using other stressors or oxidants are needed to further understand of this relationship.

References

Chen, C.-A., Wang, T.-Y., Varadharaj, S., Reyes, L.A., Hemann, C., Talukder, M.A.H., Chen, Y.-R., Druhan, L.J., and Zweier, J.L. (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 468, 1115–1118.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Du, Y., Navab, M., Shen, M., Hill, J., Pakbin, P., Sioutas, C., Hsiai, T.K., and Li, R. (2013). Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. 436, 462–466.

Galougahi, K.K., Liu, C.-C., Gentile, C., Kok, C., Nunez, A., Garcia, A., Fry, N.A.S., Davies, M.J., Hawkins, C.L., Rasmussen, H.H., et al. (2014). Glutathionylation Mediates Angiotensin II–Induced eNOS Uncoupling, Amplifying NADPH Oxidase-Dependent Endothelial Dysfunction. J. Am. Heart Assoc. 3, e000731.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Zweier, J.L., Chen, C.-A., and Druhan, L.J. (2011). S-glutathionylation reshapes our understanding of endothelial nitric oxide synthase uncoupling and nitric oxide/reactive oxygen species-mediated signaling. Antioxid. Redox Signal. 14, 1769–1775.

Decrease, GTPCH-1 leads to Decrease, Tetrahydrobiopterin (https://aopwiki.org/relationships/952)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Strong**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The relationship between GTPCH-1 and BH4 is supported in humans (Jayaram et al., 2015), cows (Abdelghany et al., 2017; Whitsett et al., 2007, Wang et al., 2008), mice (Adlam et al., 2012; Chuaiphichai et al., 2014; Crabtree et al., 2009; Tatham et al., 2009; Wang et al., 2008) and rats (Cervantes-Pérez et al., 2012).

How Does This Key Event Relationship Work

Guanosine triphosphate cyclohydrolase-1 (GTPCH-1) is the rate-limiting enzyme in the de novo biosynthesis of BH4, which is an essential cofactor for eNOS and NO generation (Wang et al., 2008). Oxidative stress can disrupt and decrease GTPCH-1 activity, leading to decreased BH4 levels and subsequent uncoupling of eNOS.

Weight of Evidence

Biological Plausibility

As GTPCH-1 is required for BH4 biosynthesis, there is strong biological plausibility for this relationship.

Many studies demonstrated that deletion of GTPCH-1 led to the deficiency of BH4 in endothelial cells. In the hph-1 mouse model, cardiac GTPCH-1 enzymatic activity was reduced by 90% compared to wild-type mice, which led to 60% reduction in BH4 levels (Adlam et al., 2012). In another mouse model of endothelial-targeted deletion of GTPCH-1, BH4 levels in lung, heart and aorta were significantly decreased compared to wild-type mice (Chuaiphichai et al., 2014). GTPCH-1 siRNA significantly reduced GTPCH-1 enzyme activity and BH4 levels in murine sEnd.1 and aortic endothelial cells (Crabtree et al., 2009; Tatham et al., 2009; Wang et al., 2008). The selective GTPCH-1 inhibitor diaminohydroxypyrimidine (DAHP) reduced levels of BH4 in bovine aortic endothelial cells (BAECs) (Wang et al., 2008). Also, transgenic overexpression of GTPCH-1 increased BH4 protein levels in murine hearts and aortas, leading to enhanced eNOS activity (Alp et al., 2003; Carnicer et al., 2012).

Empirical Support for Linkage

Include consideration of temporal concordance here

Exposure to a wide range of stimuli led to a decrease in both GTPCH-1 expression and activity and a decrease in BH4 levels, indicating strong empirical support between these two key events. An assumption that a decrease in GTPCH-1 expression and activity also results in a decrease in BH4 levels was made for these studies.

After myocardial reperfusion, GTPCH-1 activity decreased from 100% to 50% and arterial BH4 levels was reduced by 32% (Jayaram et al., 2015).

IL6/TNF/LPS (4 ng/mL, 10 nmol/L, 80 ng/mL) stimulation for 24 hours induced a 3-fold upregulation of GTPCH-1 gene expression and 3- to 4-fold increase in vascular BH4 (Antoniades et al., 2011). For this evidence, an assumption that a change in GTPCH-1 gene expression would affect GTPCH-1 enzyme activity was made.

Cigarette smoke extract (CSE, 5%) exposure for 4 hours significantly decreased BH4 levels from 100% to 50% and GTPCH-1 expression from 100% to 51%, while a lower concentration of 2.5% CSE did not cause a significant change in bovine aortic endothelial cells (Abdelghany et al., 2017).

In BAECs, stimulation with 25 μM 4-hydroxy-2-nonenal decreased BH4 levels from 10 pmol/mg protein to 6.1 pmol/mg protein and GTPCH-1 activity from 27 pmol/hr/mg protein to 23 pmol/hr/mg protein (Whitsett et al., 2007).

In a rat hypertensive model, the decrease in GTPCH-1 protein expression (via densitometry of western blot band intensity, 0.089 control to 0.0087) and BH4 (33.71±3.318 pmol control to 17.144±2.251 pmol) induced by aortic coarctation was attenuated by clofibrate treatment (GTPCH-1: 0.088±0.022 band intensity, BH4: 32.534±5.809 pmol) (Cervantes-Pérez et al., 2012).

Uncertainties or Inconsistencies

There are no uncertainties or inconsistencies.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Based on the relationship between GTPCH-1 and BH4, it would be possible that any change in GTPCH-1 activity would affect BH4 biosynthesis. The studies above showed that there are many modulators of the response-response relationships including cardiac reperfusion (Jayaram et al., 2015), cytokines (Antoniades et al., 2011), CSE (Abdelghany et al., 2017), and 4-hydroxy-2-nonenal (Whitsett et al., 2007).

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review).

Adlam, D., Herring, N., Douglas, G., De Bono, J.P., Li, D., Danson, E.J., Tatham, A., Lu, C.-J., Jennings, K.A., Cragg, S.J., et al. (2012). Regulation of β-adrenergic control of heart rate by GTP-cyclohydrolase 1 (GCH1) and tetrahydrobiopterin. Cardiovasc. Res. 93, 694–701.

Alp, N.J., Mussa, S., Khoo, J., Cai, S., Guzik, T., Jefferson, A., Goh, N., Rockett, K.A., and Channon, K.M. (2003). Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. J. Clin. Invest. 112, 725–735.

Antoniades, C., Cunnington, C., Antonopoulos, A., Neville, M., Margaritis, M., Demosthenous, M., Bendall, J., Hale, A., Cerrato, R., Tousoulis, D., et al. (2011). Induction of vascular GTP-cyclohydrolase I and endogenous tetrahydrobiopterin synthesis protect against inflammation-induced endothelial dysfunction in human atherosclerosis. Circulation 124, 1860–1870.

Carnicer, R., Hale, A.B., Suffredini, S., Liu, X., Reilly, S., Zhang, M.H., Surdo, N.C., Bendall, J.K., Crabtree, M.J., Lim, G.B.S., et al. (2012). Cardiomyocyte GTP cyclohydrolase 1 and tetrahydrobiopterin increase NOS1 activity and accelerate myocardial relaxation. Circ. Res. 111, 718–727.

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Chen, C.-A., Lin, C.-H., Druhan, L.J., Wang, T.-Y., Chen, Y.-R., and Zweier, J.L. (2011). Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. J. Biol. Chem. 286, 29098–29107.

Chuaiphichai, S., McNeill, E., Douglas, G., Crabtree, M.J., Bendall, J.K., Hale, A.B., Alp, N.J., and Channon, K.M. (2014). Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 64, 530–540.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Tatham, A.L., Crabtree, M.J., Warrick, N., Cai, S., Alp, N.J., and Channon, K.M. (2009). GTP cyclohydrolase I expression, protein, and activity determine intracellular tetrahydrobiopterin levels, independent of GTP cyclohydrolase feedback regulatory protein expression. J. Biol. Chem. 284, 13660–13668.

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Whitsett, J., Picklo, M.J., and Vasquez-Vivar, J. (2007). 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation. Arterioscler. Thromb. Vasc. Biol. 27, 2340–2347.

Decrease, Tetrahydrobiopterin leads to Uncoupling, eNOS (https://aopwiki.org/relationships/953)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Strong**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The relationship between BH4 depletion and eNOS uncoupling was observed in humans (Jayaram et al., 2015), cows (Wang et al., 2008; Whitsett et al., 2007), mice (Chuaiphichai et al., 2014; Crabtree et al., 2009) and rats (Cervantes-Pérez et al., 2012; Dumitrescu et al., 2007).

How Does This Key Event Relationship Work

Oxidative stress leads to the excessive oxidation and depletion of BH4, resulting in eNOS uncoupling where eNOS produces superoxide rather than nitric oxide (Förstermann and Münzel, 2006).

Weight of Evidence

Biological Plausibility
BH4 is an essential cofactor for eNOS and is required for its enzymatic activity to produce NO. The depletion of BH4 leading to eNOS uncoupling is well-studied, thus there is strong biological plausibility for this link.

Two mouse studies showed limited BH4 availability induced eNOS uncoupling by reducing eNOS activity, leading to decreased nitric oxide and increased superoxide. In the mouse endothelial cell line sEnd.1, BH4 deficiency induced eNOS uncoupling as determined by superoxide production and impaired vasodilation (Crabtree et al., 2009). In primary aortic endothelial cells of GTPCH1-knockout mice, BH4 depletion significantly reduced eNOS activity, increased basal superoxide production and decreased NO bioactivity (Chuaiphichai et al., 2014). In rat hearts, BH4 content and eNOS activity were decreased in a time-dependent manner following myocardial ischemia with a marked decline after thirty minutes, while superoxide generation increased (Dumitrescu et al., 2007).

BAECs undergoing hypoxia and reoxygenation had decreased BH4 and decreased NO production, which was partially restored by treatment with the xanthine oxidase inhibitor oxypurinol, N-acetyl-l-cysteine (NAC) and NAC+BH4 (De Pascali et al., 2014). Inhibition of BH4 due to treatment with 4-HNE decreased eNOS activity and NO production in BAECs (Whitsett et al., 2007).

Many studies demonstrated that BH4 treatment reduced eNOS-mediated superoxide generation and increased NO formation in bovine, mouse, and rat endothelium (Chen et al., 2011; De Pascali et al., 2014; Landmesser et al., 2003; Ozaki et al., 2002; Shinozaki et al., 2000). Clinical studies reported improvement endothelial function in cardiovascular disease after treatment with BH4 (Wang et al., 2014). Oral treatment with BH4 in hypertensive patients significantly decreased blood pressure.

Empirical Support for Linkage
Include consideration of temporal concordance here

Multiple studies demonstrated a strong dependency between BH4 and eNOS uncoupling; decreased BH4 along with decreased eNOS activity, decreased NO production or increased superoxide generation were observed following various perturbations.

Prolonged myocardial ischemia (>30 min) in isolated rat hearts caused extensive BH4 depletion (95% depletion), which was paralleled by decreased eNOS activity (58% reduction) and increased superoxide generation from <0.01 relative fluorescence unit/mg protein to 0.3 measured using a fluorescence detector-based assay (Dumitrescu et al., 2007). Similarly, cardiac reperfusion patients exhibited a reduction in BH4 levels by 32%, decreased eNOS activity by 40% and increased superoxide production from 37.83 to 65.02 light unit/s/mg as detected using lucigenin-enhanced chemiluminescence (Jayaram et al., 2015).

In BAECs treated with 10 mmol/L DAHP or 25 μM 4-HNE for 4 hours, BH4 levels decreased (control: 10 pmol/mg, DAHP: 4.8 pmol/mg, 4-HNE: 6.1 pmol/mg). At 24 hours, a reduction in NO (control: 1678 pmol/mg, DAHP: 1274 pmol/mg, 4-HNE: 1106 pmol/mg) and increased superoxide formation (control: 59 pmol/mg, DAHP: 97 pmol/mg, 4-HNE: 122 pmol/mg) were observed (Whitsett et al., 2007). Another study in BAECs using DAHP showed similar results for BH4 (control: 20.5 pmol/mg, DAHP: 11.8 pmol/mg), superoxide (control: 100%, DAHP: 257%), and NO (control: 96%, DAHP: 60%) (Wang et al., 2008).

In BAECs undergoing hypoxia and reoxygenation (H/R), treatment with oxypurinol increased BH4 levels from 6.1 ± 0.9 pmol/mg protein (after H/R) to 11.9 ± 0.8 pmol/mg protein and increased NO from 34.2 ± 1.7% to 63.7 ± 3.0%, demonstrating that these key events are modulated together (De Pascali et al., 2014).

In a rat model of hypertension, BH4 depletion and eNOS uncoupling induced by aortic coarctation (AoCo) was reversed by treatment with clofibrate; eNOS activity increased from 6.927±3.475 ng L-citrulline/mg protein/30 min to 23.2 ± 9.034 ng L-citrulline/mg protein/30 min, BH4 levels increased from 17.144±2.251 pmol to 32.534±5.809 pmol, and superoxide decreased from relative fluorescence intensity of 32.22±2.903 to 24.59±1.124 (Cervantes-Pérez et al., 2012). Note that normal eNOS activity converts L-arginine to L-citrulline and produces NO; thus L-citrulline is an indirect measure of eNOS activity.

Uncertainties or Inconsistencies
The uncoupling of eNOS may also occur through other mechanisms such as S-glutathionylation of eNOS and depletion of L-arginine (Zweier et al., 2011).

Quantitative Understanding of the Linkage
Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

As BH4 is required for normal eNOS function, it could be possible that any change in BH4 may affect eNOS function. The studies above demonstrated that there are many modulators of the response-response relationships including cardiac reperfusion (Jayaram et al., 2015), DAHP (Wang et al., 2008; Whitsett et al., 2007), and 4-HNE (Whitsett et al., 2007).

References

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Chen, C.-A., Lin, C.-H., Druhan, L.J., Wang, T.-Y., Chen, Y.-R., and Zweier, J.L. (2011). Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. J. Biol. Chem. 286, 29098–29107.

Chuaiphichai, S., McNeill, E., Douglas, G., Crabtree, M.J., Bendall, J.K., Hale, A.B., Alp, N.J., and Channon, K.M. (2014). Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 64, 530–540.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Förstermann, U., and Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E., and Harrison, D.G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest. 111, 1201–1209.

Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui, H., Sakurai, H., Yoshida, Y., et al. (2002). Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. J. Clin. Invest. 110, 331–340.

Shinozaki, K., Nishio, Y., Okamura, T., Yoshida, Y., Maegawa, H., Kojima, H., Masada, M., Toda, N., Kikkawa, R., and Kashiwagi, A. (2000). Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ. Res. 87, 566–573.

Wang, Q., Yang, M., Xu, H., and Yu, J. (2014). Tetrahydrobiopterin improves endothelial function in cardiovascular disease: a systematic review. Evid.-Based Complement. Altern. Med. ECAM 2014, 850312.

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Whitsett, J., Picklo, M.J., and Vasquez-Vivar, J. (2007). 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation. Arterioscler. Thromb. Vasc. Biol. 27, 2340–2347.

Uncoupling, eNOS leads to Depletion, Nitric Oxide (https://aopwiki.org/relationships/956)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Strong**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The relationship between NO depletion and eNOS uncoupling was observed in humans, cows, and rats as demonstrated by the above studies.

How Does This Key Event Relationship Work

The uncoupling of eNOS occurs through a number of mechanisms like BH4 depletion or S-glutathionylation of eNOS and leads to a reduction in NO bioavailability and an elevation in superoxide production, contributing to endothelial dysfunction (Zweier et al., 2011).

Weight of Evidence

Biological Plausibility
It is well-established that uncoupling of eNOS causes eNOS to switch from producing NO to generating superoxides (Förstermann and Münzel, 2006). Many studies that reported BH4 depletion leading to eNOS uncoupling (https://aopwiki.org/wiki/index.php/Relationship:953) or S-glutathionylation leading to eNOS uncoupling (https://aopwiki.org/wiki/index.php/Relationship:955) measured levels of NO and superoxide which are indicative of eNOS uncoupling.

Empirical Support for Linkage
Include consideration of temporal concordance here

Multiple experiments demonstrated that eNOS uncoupling results in increased superoxide formation and decreased NO production, which provide strong empirical support for this KER.

Prolonged myocardial ischemia (>30 min) in isolated rat hearts decreased eNOS activity (58% reduction) and increased superoxide generation from <0.01 relative fluorescence unit/mg to 0.3 as measured using a fluorescence detector-based assay (Dumitrescu et al., 2007). Similarly, cardiac reperfusion patients had decreased eNOS activity (40% reduction) and increased superoxide production from 37.83 to 65.02 light unit/s/mg as detected using lucigenin-enhanced chemiluminescence (Jayaram et al., 2015).

In BAECs, treatment with 10 mmol/L DAHP or 25 µM 4-HNE for 24 hours led to a reduction in NO (control: 1678 pmol/mg, DAHP: 1274 pmol/mg, 4-HNE: 1106 pmol/mg) and increased superoxide formation (control: 59 pmol/mg, DAHP: 97 pmol/mg, 4-HNE: 122 pmol/mg) (Whitsett et al., 2007). Similar results were observed in another study using DAHP-treated BAECs; superoxide was increased (control: 100%, DAHP: 257%) and NO was decreased (control: 96%, DAHP: 60%) (Wang et al., 2008).

In BAECs undergoing hypoxia and reoxygenation (H/R), NO decreased from 34.2±1.7% to 63.7±3.0% and superoxide increased from relative intensity of 3.5 to 49.2, demonstrating that these key events are modulated together (De Pascali et al., 2014).

BCNU (25 µM, 80 µM) resulted in increased superoxide (25 µM: 432 fluorescence intensity, 80 µM:759 fluorescence intensity) and decreased NO (25 µM: 61%, 80 µM:36%) in a dose-dependent manner in BAECs (Chen et al., 2010).

Exposure to 50 µmol/L peroxynitrite for 3 hours led to an increase in superoxide from 2 nmol/min/well to 8.6 nmol/min/well and a decrease in NO from 90% to 6.5% in BAECs (Zou et al., 2002).

Uncertainties or Inconsistencies
There are no uncertainties or inconsistencies.

Quantitative Understanding of the Linkage
Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

The uncoupling of eNOS automatically results in NO depletion and increased superoxide production. The experimental studies above included a number of modulators of the response-response relationship, such as peroxynitrite, BCNU, and DAHP.

References

Chen, X., Xu, J., Feng, Z., Fan, M., Han, J., and Yang, Z. (2010). Simvastatin combined with nifedipine enhances endothelial cell protection by inhibiting ROS generation and activating Akt phosphorylation. Acta Pharmacol. Sin. 31, 813–820.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Förstermann, U., and Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Whitsett, J., Picklo, M.J., and Vasquez-Vivar, J. (2007). 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation. Arterioscler. Thromb. Vasc. Biol. 27, 2340–2347.

Zou, M.-H., Hou, X.-Y., Shi, C.-M., Nagata, D., Walsh, K., and Cohen, R.A. (2002). Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J. Biol. Chem. 277, 32552–32557.

Zweier, J.L., Chen, C.-A., and Druhan, L.J. (2011). S-glutathionylation reshapes our understanding of endothelial nitric oxide synthase uncoupling and nitric oxide/reactive oxygen species-mediated signaling. Antioxid. Redox Signal. 14, 1769–1775.

Depletion, Nitric Oxide leads to Impaired, Vasodilation (https://aopwiki.org/relationships/958)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9986)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

This relationship between NO depletion and impaired vasodilation was shown in humans (Li et al. 1993, Heitzer et al. 2000), rabbits (Luo et al. 2000), mice (Luo et al. 2000, Wang et al. 2008) and rats (Paulis et al. 2008, Li et al. 1993, Sélley et al. 2014, Chen et al. 2010).

How Does This Key Event Relationship Work

Nitric oxide (NO) is a critical endothelium-derived hyperpolarising factor (EDHF), responsible for relaxation of vascular smooth muscle and vasodilation. The primary regulator of endothelial vasodilator function via NO is vascular shear; the frictional force exerted on the vascular wall during the flow of blood through the vessel. Vascular shear opens calcium channels on endothelial cells, and leads to the calcium-dependent activation of eNOS and thus NO production. NO then diffuses to the underlying vascular smooth muscle, where it activates soluble guanylate cyclase, causing an increase in cyclic guanosine monophosphate (cGMP), potassium ion efflux, hyperpolarization and smooth muscle relaxation (Giles et al. 2012).

Depletion of vascular NO bioavailability causes an imbalance in the maintenance of vascular tone, which shifts in favour of vasoconstriction, and hence elevates blood pressure (Kojda et al. 1999). Under oxidative stress, decreased NO bioavailability results in impaired endothelium-dependent vasodilation (Silva et al., 2012).

Weight of Evidence

Biological Plausibility

Vasodilation is caused by the relaxation of vascular smooth muscle cells within the walls of blood vessels, which is regulated through a number of mechanisms, including cyclic GMP-dependent hyperpolarization and relaxation via NO. Thus, alterations to NO levels have an influence on vasodilation (Silva et al., 2012). Many animal studies demonstrated that inhibition of NO via eNOS inhibitors impaired acetylcholine-induced endothelium-dependent vasodilation, providing strong biological plausibility for this key event relationship.

Constitutively active AKT, which has been shown to phosphorylate and activate eNOS, increased resting vessel diameter in a rabbit femoral artery model, but infusion with eNOS inhibitor L-NAME reversed this effect (Luo et al., 2000). Dominant negative AKT, which prevents eNOS activation, also blocked vasodilation in response to the endothelium-dependent agonist acetylcholine (ACh) in rabbit arteries and mouse aortas. Since acetylcholine induces endothelium-dependent vasodilation, the assumption is that when eNOS inhibitors blocks Ach-induced vasodilation, NO production is decreased and vasodilation is impaired. L-NAME abolished endothelium-dependent relaxations induced by ACh in aortas of wild-type and hph-1 mice (Li et al., 2007). ACh induced a dose-dependent endothelium-dependent relaxation in rat small mesenteric arteries, but the NO-dependent component of ACh-induced relaxation was decreased in L-NAME-treated rats (Paulis et al., 2008). After L-NAME cessation, the NO-dependent component was restored to above control levels. L-NAME diminished eNOS activity by 66% and its cessation restore NOS activity to control levels in rat aortas. Another study showed that L-NAME caused a mild but significant decrease in vasodilation in rat thoracic aorta (Sélley et al., 2014).

Empirical Support for Linkage

Include consideration of temporal concordance here

There is moderate empirical support for depletion of NO leading to impaired vasodilation based on several studies measuring decreased NO and vasodilation following a number of perturbations. In rat small mesenteric arteries, treatment with L-NAME for five weeks decreased NO-dependent relaxation from 28% to 7.5%, while diminishing eNOS activity by 66% (Paulis et al, 2008). In rat aortic rings, BCNU treatment for 4 hours caused a dose-dependent decrease in NO production (control: 100%, 25 µM: 61%, 80 µM: 36%),

and also decreased vasodilation from 69% control to 37% (at 80 µM) (Chen et al., 2010). Similarly, treatment with 10 mM DAHP for 24 hours caused a decrease in both NO (from 100% to 61%) and vasodilation (from 87% to 42%) (Wang et al., 2008).

In humans, application of L-NAME and/or N^G-monomethyl-L-arginine (L-NMMA) resulted in elevated mean arterial pressure via impairment of NO-mediated vasodilation. Application of both drugs compounded the effect (Sander et al. 1999). BH4 administration reversed NO depletion by L-NMMA and improved forearm venous blood flow in chronic smokers (Heizter et al. 2000). In human skin, acetylcholine-mediated vasodilation was shown to be modulated by NO and impaired by the eNOS inhibitor; L-NAME (Kellogg et al. 2005). Intravenous infusion of the eNOS substrate L-arginine was shown to decrease blood pressure and total peripheral resistance. The onset and the duration of the vasodilator effect of L-arginine and its effects on endogenous NO production closely corresponded to the plasma concentration half-life of l-arginine, and was associatedwith a concomminant elevation of urinary nitrate and cyclic GMP excretion (Bode-Böger et al. 1998). Finally, in chronic smokers with impaired flow-mediated dilation (FMD; 5.6±3.0% vs. 8.1±3.7% for non-smokers P<0.01), administration of the essential eNOS cofactor; BH4, improved FMD in both cohorts (6.6±3.3% vs. 9.8±3.2%; P<0.01) and smoking cessation for 1 week also improved FMD (from 5.0±2.9 to 7.8±3.2%;P<0.01). This data indicates that improvement in arterial vasodilation was partially improved by BH4 administration (Taylor et al. 2016). Another study by Carnevale et al. (2016) in which current smokers switched to an e-cigarette product for 1 week showed significant reductions in FMD impairment compared to baseline, which was associated with improvement in levels of 8-iso-prostagladin F2α (a biomarker of oxidative stress).

Uncertainties or Inconsistencies

While the effect of NO depletion on impaired vasodilation is clear, the NO pathway does not appear to be solely responsible for this phenomenon. Vascular tone is a balance between relaxation and constriction factors. Studies have shown that when NO bioavailability is decreased, COX-mediated pro-inflammatory factors such as prostaglandins (Kellogg et al. 2005, Lüscher and Vanhoutte 1986) and Endothelin-1 (Taddei et al. 2003) contribute to a shift in vascular tone towards vasoconstiction. The review by Silva et al. (2012) discusses the roles of various pathways and their effects on vascular tone. The relative contribution of these mechanisms towards vascular tone is currently unknown.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

In rat aortic rings, BCNU treatment for 4 hours caused a dose-dependent decrease in NO production (control: 100%, 25 µM: 61%, 80 µM: 36%), and also decreased vasodilation from 69% control to 37% (at 80 µM) (Chen et al., 2010). Similarly, treatment with 10 mM DAHP for 24 hours caused a decrease in both NO (from 100% to 61%) and vasodilation (from 87% to 42%) (Wang et al., 2008).

Hence, it appears that a decrease of 40% NO relative to baseline conditions appears sufficient to impact vasodilation since the experiments show that NO decreases to near 60% after applying the stressors. The studies used several perturbations that were able to modulate NO production and vasodilation simultaneously including L-NAME, DAHP and BCNU. The evidence is qualitative however.

Furthermore, as NO is a highly volatile substance, researchers are not able to treat models/subjects with specific doses to observe the corresponding biological effects. NO donors such as sodium nitroprusside and glyceryl trinitrate are often used to generate NO in humans and animal studies, however this is indepedent of the vascular endothelium. More stable metabolites of NO are often measured in vivo to estimate NO turnover e.g. Nitrate/nitrite, however the conversion kinetics of NO to these metabolites is unclear and hence limited to qualitative comparisons. An example is the study by Bode-Böger et al. (1998) which investigated the pharmacokinetics of the eNOS substrate L-arginine and its subsequent effects upon vasodilation (measured using total peripheral resistance and blood pressure as surrogate endpoints). Plasma l-arginine levels increased to (mean±s.e.mean) 6223±407 (range, 5100–7680) and 822±59 (527–955) µmol l−1 after intravenous infusion of 30 g and 6 g l-arginine, respectively, and to 310±152 (118–1219) µmol l−1 after oral ingestion of 6 g l-arginine. Oral bioavailability of l-arginine was 68±9 (51–87)%. Clearance was 544±24 (440–620), 894±164 (470–1190), and 1018±230 (710–2130) ml min−1, and elimination half-life was calculated as 41.6±2.3 (34–55), 59.6±9.1 (24–98), and 79.5±9.3 (50–121) min, respectively, for 30 g i.v., 6 g i.v., and 6 g p.o. of l-arginine. Blood pressure and total peripheral resistance were significantly decreased after intravenous infusion of 30 g l-arginine by 4.4±1.4% and 10.4±3.6%, respectively, but were not significantly changed after oral or intravenous administration of 6 g l-arginine. l-arginine (30 g) also significantly increased urinary nitrate and cyclic GMP excretion rates by 97±28 and 66±20%, respectively. After infusion of 6 g l-arginine, urinary nitrate excretion also significantly increased, (nitrate by 47±12% [P<0.05], cyclic GMP by 67±47% [P=ns]), although to a lesser and more variable extent than after 30 g of l-arginine. The onset and the duration of the vasodilator effect of l-arginine and its effects on endogenous NO production closely corresponded to the plasma concentration half-life of l-arginine, as indicated by an equilibration half-life of 6±2 (3.7–8.4) min between plasma concentration and effect in pharmacokinetic-pharmacodynamic analysis, and the lack of hysteresis in the plasma concentration-versus-effect plot.

References

Bode-Böger SM, Böger RH, Galland A, Tsikas D, Frölich JC. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamicrelationship. Br J Clin Pharmacol. 1998, 46(5):489-97.

Carnevale R, Sciarretta S, Violi F et al. Acute Impact of Tobacco vs Electronic Cigarette Smoking on Oxidative Stress and Vascular Function. Chest. (2016) 150(3):606-12.

Chen, C.-A., Wang, T.-Y., Varadharaj, S., Reyes, L.A., Hemann, C., Talukder, M.A.H., Chen, Y.-R., Druhan, L.J., and Zweier, J.L. (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 468, 1115–1118.

Giles TD, Sander GE, Nossaman BD et al. Impaired vasodilation in the pathogenesis of hypertension: focus on nitric oxide, endothelial-derived hyperpolarizing factors, and prostaglandins. J Clin Hypertens (2012) 14(4):198-205.

Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T, Münzel T. Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers : evidence for a dysfunctional nitric oxide synthase. Circ Res. 2000, 86(2):E36-41.

Kellogg DL Jr, Zhao JL, Coey U, Green JV. Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. J Appl Physiol (1985). 2005, 98(2):629-32.

Kojda G, Laursen JB, Ramasamy S et al. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. Cardiovasc Res. (1999) 42(1):206-13.

Li, J., Zhou, Z., Jiang, D.-J., Li, D., Tan, B., Liu, H., and Li, Y.-J. (2007). Reduction of NO- and EDHF-mediated vasodilatation in hypertension: role of asymmetric dimethylarginine. Clin. Exp. Hypertens. N. Y. N 1993 29, 489–501.

Lüscher T. F., Vanhoutte P. M. (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. Hypertension 8, 344–348

Luo, Z., Fujio, Y., Kureishi, Y., Rudic, R.D., Daumerie, G., Fulton, D., Sessa, W.C., and Walsh, K. (2000). Acute modulation of endothelial AKT/PKB activity alters nitric oxide–dependent vasomotor activity in vivo. J. Clin. Invest. 106, 493–499.

Paulis, L., Zicha, J., Kunes, J., Hojna, S., Behuliak, M., Celec, P., Kojsova, S., Pechanova, O., and Simko, F. (2008). Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor. Hypertens. Res. Off. J. Jpn. Soc. Hypertens. 31, 793–803.

Sander M, Chavoshan B, Victor RG. A large blood pressure-raising effect of nitric oxide synthase inhibition in humans. Hypertension. (1999) 33(4):937-42.

Sélley, E., Kun, S., Szijártó, I.A., Laczy, B., Kovács, T., Fülöp, F., Wittmann, I., and Molnár, G.A. (2014). Exenatide induces aortic vasodilation increasing hydrogen sulphide, carbon monoxide and nitric oxide production. Cardiovasc. Diabetol. 13, 69.

Silva, B.R., Pernomian, L., and Bendhack, L.M. (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. Front. Physiol. 3, 441.

Taddei S., Ghiadoni L., Virdis A., Versari D., Salvetti A. (2003). Mechanisms of endothelial dysfunction: clinical significance and preventive non-pharmacological therapeutic strategies. Curr. Pharm. Des. 9, 2385–2402

Taylor BA, Zaleski AL, Dornelas EA et al. The impact of tetrahydrobiopterin administration on endothelial function before and after smoking cessation in chronic smokers. Hypertens Res. (2016) 39(3):144-50.

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Impaired, Vasodilation leads to Increase, Vascular Resistance (https://aopwiki.org/relationships/982)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Weak

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

This relationship between impaired vasodilation and SVR was shown in human and rat studies.

How Does This Key Event Relationship Work

Vasodilation decreases systemic vascular resistance (SVR; also known previously as Total Peripheral Resistance; TPR), the resistance to blood flow offered by the peripheral circulation, and blood pressure through relaxation of vascular smooth muscle cells (VSMCs) (Siddiqui, 2011). When vasodilation is impaired due to decreased NO availability, SVR and blood pressure become elevated.

Weight of Evidence

The overall weight of evidence for the KER was rated "moderate" due the fact that acute pharmacological manipulation of the NO pathway resulted in corresponding changes in SVR. However, in the context of the development of hypertension, the chronic effects of impaired vasodilation are much less clear.

Biological Plausibility

It is well-accepted that vasodilation and SVR are negatively correlated; blood flow is increased when blood vessels dilate due to a decrease in vascular resistance (Siddiqui, 2011). When vasodilation is impaired, SVR increases, in turn increasing blood pressure. Agents that cause hyperpolarization are potent vasodilators and activate potassium channels, while factors causing depolarization increase vascular tone (Nelson, 1990). Vascular tone is governed by the contractile activity of VSMCs in the walls of small arteries and arterioles, and is the major determinant of the resistance to blood flow through the circulation (Jackson, 2000). VSMCs from hypertensive animals have decreased functional voltage-gated potassium channels, which may contribute to depolarization. Two studies demonstrated that blockade of potassium channels completely inhibited NO-dependent vasodilation and increased SVR (Dessy et al., 2004; Berg et al., 2011). Inhibitors of eNOS activity (L-NAME, L-NMMA), which have been shown to decrease acetylcholine-induced vasorelaxation in animal studies (Li et al., 2007; Paulis et al., 2008), also caused an increase in SVR in human studies (Wilkinson et al., 2002; McVeigh et al., 2001; Brett et al., 1998). Overall, these results provide strong biological plausibility for this link.

Empirical Support for Linkage

Include consideration of temporal concordance here

No direct evidence was found for this linkage; thus the empirical support is weak. One study indirectly showed a relationship between NO depletion and SVR, where infusion of L-NMMA (1.0 mg/kg/min) caused an increase in SVR by 63% and a 65% reduction in NO in 11 healthy volunteers after three minutes (Stamler et al., 1994). In rat small mesenteric arteries, treatment with L-NAME for five weeks decreased NO-dependent relaxation from 30% to less than 10%, but increased systolic and diastolic blood pressure to 26% and 40%, respectively (Paulis et al, 2008). Meta-analysis of clinical trials showing that infusion of the NO donor sodium nitroprusside led to a dose-dependent reduction in SVR (Eugene et al., 2016). In a study of 400 post-menopausal women with mild-moderate hypertension and impaired FMD, anti-hypertensive therapy for 6 months was shown to improve FMD by >10% in 250 women, whereas in 150 women, FMD did not significantly change (<10% change, Modena et al. 2002). Whilst the study demonstrates a linkage between vasodilation and hypertension in a large proportion of the study population, it raises a question regarding causality i.e. did NO-independent medication lower blood pressure, which in turn improved FMD? Furthermore, over a third of the population showed no change. It is possible that the medications used directly influenced key components of the NO (or other) pathways. Since the study did not elaborate on the medicatons used by the study cohort, this is impossible to determine.

Uncertainties or Inconsistencies

As mentioned above, acute pharmacological manipulation of the NO pathway results in expected changes in SVR. However, the link between chronically impaired vasodilation and SVR (the context of this AOP) is much less clear due to gaps in the literature. Epidemiological studies tend to investigate linkages between impaired vasodilation and cardiovascular events, as opposed to SVR and/or hypertension - making assessment of this KER difficult.

Furthermore, the complexity in the mechanisms influencing vascular re-modelling over time has hampered understanding of the phenomenon to date. The study by Modena et al. 2002 highlights that members of the general population respond differently to hypertensive therapy in the context of FMD improvement.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

There is very limited quantitative understanding of this linkage, however the evidence is directed towards "cardiovascular events", as opposed to specifically SVR and/or hypertension. A meta analysis by Ras et al. (2013) stated that "a 1% increase in FMD corresponds to a 14% decrease in future CVD events.". A total of 23 studies including 14,753 subjects were eligible for inclusion in the meta-analysis. For studies reporting continuous risk estimates, the pooled overall CVD risk was 0.92 (95%CI: 0.88; 0.95) per

1% higher FMD. The observed association seemed stronger (P-value<0.01) in diseased populations than in asymptomatic populations (0.87 (95%CI: 0.83; 0.92) and 0.96 (95%CI: 0.92; 1.00) per 1% higher FMD, respectively). For studies reporting categorical risk estimates, the pooled overall CVD risk for high vs. low FMD was similar in both types of populations, on average 0.49 (95%CI: 0.39; 0.62).

Similarly, Yeboah et al. (2007) concluded that FMD was a predictor of future cardiovascular (CVD) events and that systolic blood pressure per unit S.D. was a signficant (p=0.02) risk factor. However, they reported that FMD added little to current risk prediction scores for future CVD events.

References

Berg, T., and Jensen, J. (2011). Simultaneous parasympathetic and sympathetic activation reveals altered autonomic control of heart rate, vascular tension, and epinephrine release in anesthetized hypertensive rats. Front. Neurol. 2, 71.

Brett, S.E., Cockcroft, J.R., Mant, T.G., Ritter, J.M., and Chowienczyk, P.J. (1998). Haemodynamic effects of inhibition of nitric oxide synthase and of L-arginine at rest and during exercise. J. Hypertens. 16, 429–435.

Dessy, C., Moniotte, S., Ghisdal, P., Havaux, X., Noirhomme, P., and Balligand, J.L. (2004). Endothelial beta3-adrenoceptors mediate vasorelaxation of human coronary microarteries through nitric oxide and endothelium-dependent hyperpolarization. Circulation 110, 948–954.

Eugene, A.R. (2016). The influences of nitric oxide, epinephrine, and dopamine on vascular tone: dose-response modeling and simulations. Hosp. Chron. Nosokomeiaka Chron. 11, 1–8.

Jackson, W.F. (2000). Ion channels and vascular tone. Hypertension 35, 173–178.

Li, J., Zhou, Z., Jiang, D.-J., Li, D., Tan, B., Liu, H., and Li, Y.-J. (2007). Reduction of NO- and EDHF-mediated vasodilatation in hypertension: role of asymmetric dimethylarginine. Clin. Exp. Hypertens. N. Y. N 1993 29, 489–501.

McVeigh, G.E., Allen, P.B., Morgan, D.R., Hanratty, C.G., and Silke, B. (2001). Nitric oxide modulation of blood vessel tone identified by arterial waveform analysis. Clin. Sci. Lond. Engl. 1979 100, 387–393.

Modena MG, Bonetti L, Coppi F, Bursi F, Rossi R Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. J Am Coll Cardiol. 2002, 40(3):505-10.

Nelson, M.T., Patlak, J.B., Worley, J.F., and Standen, N.B. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. Am. J. Physiol. 259, C3–C18.

Paulis, L., Zicha, J., Kunes, J., Hojna, S., Behuliak, M., Celec, P., Kojsova, S., Pechanova, O., and Simko, F. (2008). Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor. Hypertens. Res. Off. J. Jpn. Soc. Hypertens. 31, 793–803.

Ras RT, Streppel MT, Draijer R, Zock PL. Flow-mediated dilation and cardiovascular risk prediction: a systematic review with meta-analysis. Int J Cardiol. 2013, 168(1):344-51.

Siddiqui, A. (2011). Effects of Vasodilation and Arterial Resistance on Cardiac Output. J. Clin. Exp. Cardiol. 02.

Stamler, J.S., Loh, E., Roddy, M.A., Currie, K.E., and Creager, M.A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 89, 2035–2040.

Wilkinson, I.B., MacCallum, H., Cockcroft, J.R., and Webb, D.J. (2002). Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br. J. Clin. Pharmacol. 53, 189–192.

Yeboah J, Crouse JR, Hsu FC, Burke GL, Herrington DM. Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. Circulation. 2007;115:2390–7.

Increase, Vascular Resistance leads to Hypertension (https://aopwiki.org/relationships/983)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adults	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

Studies supporting this key event relationship were performed in humans and rats.

How Does This Key Event Relationship Work

Hypertension is characterized partly by elevated systemic vascular resistance which is caused by alterations to vascular tone (towards vasoconstriction) over time (Lee and Griendling, 2008). As blood vessels constrict, the available volume in the vessel lumen for blood flow is restricted, resulting in elevated blood pressure.

Note : The role of the heart in the maintenace (and change) of blood pressure over time is not part of this AOP, however it is of critical importance for the development of hypertension.

Weight of Evidence

Biological Plausibility
It is well-established that increased systemic vascular resistance (SVR), increased vascular stiffness and increased vascular reactivity contribute to the pathophysiology of hypertension (Foëx and Sear, 2004; Mayet and Hughes, 2003; Brandes et al., 2014); thus biological plausibility is strong for increased SVR leading to hypertension. This is observed in patients with hypertension (Chan et al., 2016).

Empirical Support for Linkage
Include consideration of temporal concordance here

Empirical support for SVR leading to hypertension is moderate based on several human studies showing a dose-dependent change in SVR and hypertension following treatment with eNOS inhibitors L-NMMA and L-NAME. Whilst the acute changes in SVR on blood pressure in the context of endothelial NO production is well characterised, the linkage between chronically elevated SVR and hypertension is more complicated, due to the roles of the heart, nervous system and kidneys. Given that this AOP is focused on endothelial NO production, other AOPs are required to capture the roles of these other important biological processes on chronic SVR changes and hypertension, hence the rating of "moderate".

Intravenous infusion of L-NMMA (0.1, 1.0, 3.0 mg/kg/min) for 15 minutes led to a dose-dependent increase in SVR and mean arterial pressure compared to saline infusion in eight healthy men (Wilkinson et al., 2002). SVR increased from 31±2 (arbitrary units) at baseline to 57±4 at the highest dose, while mean arterial pressure increased from 86±2 mmHg baseline to 91±2 mmHg at the highest concentration. Infusion of L-NMMA (1.0 mg/kg/min) caused an increase in systolic blood pressure by 15% and SVR by 63% in 11 healthy volunteers after three minutes (Stamler et al., 1994). The study also demonstrated a dose-dependent increase in both events with five different doses of L-NMMA (0.01, 0.03, 0.1, 0.3 and 1 mg/kg/min). In another study, fifteen healthy men were intravenously infused with L-NAME at lower doses (0.25-0.75 mg/kg) for eight minutes, resulting in increased arterial pressure and SVR (McVeigh et al., 2001). Infusion with L-arginine, an eNOS substrate, restored function, specifically SVR and small artery compliance to baseline. L-NMMA (3 mg/kg) for five minutes increased mean arterial pressure by 10% and increased SVR by 46% in eight healthy subjects (Haynes et al., 1993). One experiment in rats showed that L-NAME increased SVR, aortic stiffness, and blood pressure (Nakmareong et al., 2012). Overall, these studies demonstrated that SVR and hypertension are modulated together by eNOS inhibitors.

Uncertainties or Inconsistencies
One study showed that infusion of L-NMMA (6 mg/kg) resulted in increased SVR and only a modest increase in blood pressure. Changes in diastolic blood pressure were observed to be more pronounced in healthy men, than systolic blood pressure (Brett et al., 1998), and infusion of L-arginine (an eNOS substrate) had no significant effect.

As mentioned above, other AOPs are necessary to capture understanding and assess the evidence surrounding the roles of the heart, kidney and nervous system in order to get the full picture of the linkage between chronic changes in SVR and hypertension.

Quantitative Understanding of the Linkage
Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Mean arterial pressure is calculated from cardiac output and SVR. Therefore, theoretically, any change in SVR will impact the mean arterial pressure. The studies mentioned above showed that a small change for SVR such as from 31±2 (arbitrary units) at baseline to 35 ± 2 was able to change the arterial pressure from 86±2 mmHg to 91±2 mmHg (Wilkinson et al., 2002). This trend was also observed by Stamler et al. (1994) and McVeigh et al. (2001).

References

Brandes, R.P. (2014). Endothelial dysfunction and hypertension. Hypertension 64, 924–928.

Brett, S.E., Cockcroft, J.R., Mant, T.G., Ritter, J.M., and Chowienczyk, P.J. (1998). Haemodynamic effects of inhibition of nitric oxide synthase and of L-arginine at rest and during exercise. J. Hypertens. 16, 429–435.

Chan, S.S., Tse, M.M., Chan, C.P., Tai, M.C., Graham, C.A., and Rainer, T.H. (2016). Haemodynamic changes in emergency department patients with poorly controlled hypertension. Hong Kong Med. J. Xianggang Yi Xue Za Zhi Hong Kong Acad. Med. 22, 116–123.

Foëx, P., and Sear, J.W. (2004). Hypertension: pathophysiology and treatment. Contin. Educ. Anaesth. Crit. Care Pain 4, 71–75.

Haynes, W.G., Noon, J.P., Walker, B.R., and Webb, D.J. (1993). Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. J. Hypertens. 11, 1375–1380.

Lee, M.Y., and Griendling, K.K. (2008). Redox signaling, vascular function, and hypertension. Antioxid. Redox Signal. 10, 1045–1059.

Mayet, J., and Hughes, A. (2003). Cardiac and vascular pathophysiology in hypertension. Heart Br. Card. Soc. 89, 1104–1109.

McVeigh, G.E., Allen, P.B., Morgan, D.R., Hanratty, C.G., and Silke, B. (2001). Nitric oxide modulation of blood vessel tone identified by arterial waveform analysis. Clin. Sci. Lond. Engl. 1979 100, 387–393.

Nakmareong, S., Kukongviriyapan, U., Pakdeechote, P., Kukongviriyapan, V., Kongyingyoes, B., Donpunha, W., Prachaney, P., and Phisalaphong, C. (2012). Tetrahydrocurcumin alleviates hypertension, aortic stiffening and oxidative stress in rats with nitric oxide deficiency. Hypertens. Res. Off. J. Jpn. Soc. Hypertens. 35, 418–425.

Silva, B.R., Pernomian, L., and Bendhack, L.M. (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. Front. Physiol. 3, 441.

Stamler, J.S., Loh, E., Roddy, M.A., Currie, K.E., and Creager, M.A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 89, 2035–2040.

Wilkinson, I.B., MacCallum, H., Cockcroft, J.R., and Webb, D.J. (2002). Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br. J. Clin. Pharmacol. 53, 189–192.

Decrease, AKT/eNOS activity leads to Depletion, Nitric Oxide (https://aopwiki.org/relationships/988)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Strong**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The relationship between decreased AKT/eNOS activity and NO depletion is supported by studies performed in humans, cows, and rats.

How Does This Key Event Relationship Work

AKT can phosphorylate eNOS which leads to increased eNOS enzymatic activity and subsequent NO production (Dimmeler et al., 1999; Fulton et al., 1999). Inhibition of AKT attenuates eNOS phosphorylation and its activity, resulting in decreased NO bioavailability and endothelial dysfunction.

Weight of Evidence

Biological Plausibility
Two studies demonstrated that AKT can directly phosphorylate eNOS at Ser1177/Ser1179, leading to increased eNOS enzymatic activity and subsequent NO production in COS cells (Dimmeler et al., 1999; Fulton et al., 1999). Expressing active AKT in bovine microvascular endothelial cells also induced NO release (Fulton et al., 1999). Inhibition of Akt or a mutation of eNOS at AKT-sensitive sites attenuated eNOS phosphorylation and its activity, resulting in decreased NO bioavailability and endothelial dysfunction (Dimmeler et al., 1999; Fulton et al., 1999; Uruno et al., 2005). Treatment of BAECs with SIN-1 resulted in a reduction in both eNOS activity and NO production (Das et al., 2014). Cigarette smoke extract (CSE) treatment inhibited AKT and eNOS and NO release in VEGF-stimulated HUVECs (Michaud et al., 2006), and methylglyoxal and high glucose reduced eNOS bradykinin-stimulated eNOS activity and NO production in HUVECs (Dhar et al., 2010). Overall, the biological plausibility for decreased AKT/eNOS activity leading to NO depletion is strong.

Empirical Support for Linkage
Include consideration of temporal concordance here

Empirical support for this linkage is strong since various stressors (ischemia, peroxynitrite, SIN-1, insulin+orotic acidura, etc.) can cause a decrease in AKT or eNOS activity which then leads to increased eNOS uncoupling and decreased NO levels.

Treatment of HUVECs with 30 µM methylglyoxal (MG) and 25 mM high glucose (HG) concentrations for 24 hours caused a decrease in eNOS activity (control: 100%, MG: 55%, HG: 66%), while a shorter treatment for 3 hours caused a decrease in NO production (control: 20 µM, MG: 12 µM, HG: 16 µM) (Dhar et al., 2010).

Treatment of BAECs with SIN-1 for 2 hours decreased eNOS activity from 100% to 70% and NO production from 100% to 53% (Das et al., 2014).

In HUVECs, exposure to 5 µmol/L peroxynitrite for 5 minutes reduced AKT phosphorylation by 30% (Song et al., 2007) while exposure to 50 µmol/L peroxynitrite for 3 hours reduced NO production from 90% to 6.5% (Zou et al., 2002), demonstrating that AKT phosphorylation occurs before NO production at a lower dose.

Prolonged myocardial ischemia (>30 min) in isolated rat hearts caused decreased eNOS activity (58% reduction) and increased superoxide generation (from relative fluorescence unit of <0.01 to 0.3), suggesting a depletion in NO production (Dumitrescu et al., 2007).

Treatment of HUVECs with 50 µM insulin for 30 minutes increased phosphorylation of AKT (control: 1, insulin: 5.7) and eNOS (control: 1, insulin: 1.9) as well as NO production (control: 100%, insulin: 157%), but these effects were reversed by additional treatment with 100 µM orotic aciduria (OA) for one hour (Akt: 1.8; eNOS: 0.28; NO: 106%) (Choi et al., 2015). Similar results were observed with addition of uric acid to insulin-treated HUVECs (Choi et al., 2014).

Treatment with all-trans retinoic acid (ATRA) and retinoic acid receptor agonist Am580 at 1 µmol/L for 48 hours also increased AKT (control: 100%, ATRA: 304%, Am580: 212%) and eNOS (control: 100%, ATRA: 276%, Am580: 209%) phosphorylation and NO production (control: 100%, ATRA: 170%, Am580: 169%) in human endothelial cells (Uruno et al., 2005).

Uncertainties or Inconsistencies
In the context of this AOP, decreased activity of AKT is likely due to proteasomal degradation following exposure to a (oxidative) stressor (Abdehghany et al. 2017). However, decreased eNOS activity could be due to multiple causes, highlighted in this AOP. Firstly, if AKT expression levels are reduced, it follows that eNOS phosphorylation will be reduced. Secondly, similar to AKT, eNOS itself has been shown to be susceptible to proteasomal degradation (Abdehghany et al. 2017). Thirdly, depletion of BH4 and/or S-glutathionylation has been shown to uncouple eNOS, leading to reduced NO levels and increased superoxide levels. The relative contribution of each of these eNOS perturbation routes to NO depletion is currently unknown.

Quantitative Understanding of the Linkage
Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

In two studies (Song et al, 2007; Das et al., 2014), it appears that a minimum of 30% reduction in eNOS activity or AKT phosphorylation caused a change in NO production in as little as five minutes. Other studies showed that 50-60% reduction in AKT phosphorylation/eNOS activity will lead to decreased NO bioavailability (Dhar et al., 2010; Dumitrescu et al., 2007). The studies above demonstrated that there are several known modulators for these two key events including peroxynitrite, high glucose, methylglyoxal, insulin and ATRA.

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review).

Choi, Y.-J., Yoon, Y., Lee, K.-Y., Hien, T.T., Kang, K.W., Kim, K.-C., Lee, J., Lee, M.-Y., Lee, S.M., Kang, D.-H., et al. (2014). Uric acid induces endothelial dysfunction by vascular insulin resistance associated with the impairment of nitric oxide synthesis. FASEB J. 28, 3197–3204.

Choi, Y.-J., Yoon, Y., Lee, K.-Y., Kang, Y.-P., Lim, D.K., Kwon, S.W., Kang, K.-W., Lee, S.-M., and Lee, B.-H. (2015). Orotic Acid Induces Hypertension Associated with Impaired Endothelial Nitric Oxide Synthesis. Toxicol. Sci. 144, 307–317.

Das, A., Gopalakrishnan, B., Druhan, L.J., Wang, T.-Y., De Pascali, F., Rockenbauer, A., Racoma, I., Varadharaj, S., Zweier, J.L., Cardounel, A.J., et al. (2014). Reversal of SIN-1-induced eNOS dysfunction by the spin trap, DMPO, in bovine aortic endothelial cells via eNOS phosphorylation. Br. J. Pharmacol. 171, 2321–2334.

Dhar, A., Dhar, I., Desai, K.M., and Wu, L. (2010). Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. Br. J. Pharmacol. 161, 1843–1856.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., and Sessa, W.C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597–601.

Michaud, S.E., Dussault, S., Groleau, J., Haddad, P., and Rivard, A. (2006). Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species. J. Mol. Cell. Cardiol. 41, 275–284.

Song, P., Wu, Y., Xu, J., Xie, Z., Dong, Y., Zhang, M., and Zou, M.-H. (2007). Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. Circulation 116, 1585–1595.

Song, P., Xie, Z., Wu, Y., Xu, J., Dong, Y., and Zou, M.-H. (2008). Protein kinase Czeta-dependent LKB1 serine 428 phosphorylation increases LKB1 nucleus export and apoptosis in endothelial cells. J. Biol. Chem. 283, 12446–12455.

Uruno, A., Sugawara, A., Kanatsuka, H., Kagechika, H., Saito, A., Sato, K., Kudo, M., Takeuchi, K., and Ito, S. (2005). Upregulation of nitric oxide production in vascular endothelial cells by all-trans retinoic acid through the phosphoinositide 3-kinase/Akt pathway. Circulation 112, 727–736.

Zou, M.-H., Hou, X.-Y., Shi, C.-M., Nagata, D., Walsh, K., and Cohen, R.A. (2002). Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J. Biol. Chem. 277, 32552–32557.

Peptide Oxidation leads to Decrease, AKT/eNOS activity (https://aopwiki.org/relationships/992)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Strong**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Bos taurus	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)
Mus musculus	Mus musculus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The relationship between oxidative stress and decreased AKT/eNOS activity is supported by studies performed in humans, cows, mice and rats.

How Does This Key Event Relationship Work

Exposure to known inducers of oxidative stress causes the phosphorylation of AKT and eNOS, leading to a decrease in their activities.

Weight of Evidence

Biological Plausibility
Multiple experimental studies reported modulations in Akt and eNOS phosphorylation/activity following oxidative stress, thus providing strong biological plausibility for this key event relationship.

In HUVECs, peroxynitrite significantly inhibited AKT phosphorylation at Ser473 and AKT activity (Song et al., 2007, 2008; Zou et al., 2002). However, Zou et al. (2002) found that peroxynitrite increased eNOS phosphorylation at Ser1199, but decreased NO availability, suggesting eNOS phosphorylation may not depend on AKT. Treatment of BAECs with SIN-1, a source of peroxynitrite, inhibited eNOS activity by 30% and AKT/eNOS phosphorylation (Das et al., 2014).

High glucose concentrations can also inhibit AKT phosphorylation (Song et al., 2008). HUVECs treated with methylglyoxal and high concentrations of glucose exhibited reduced bradykinin-stimulated eNOS activity via Ser1177 phosphorylation (Dhar et al., 2010), while hyperglycemia inhibited eNOS activity in BAECs (Du et al., 2001). In EA.hy926 endothelial cells, methylglyoxal treatment results in reduced eNOS phosphorylation at Ser1177 (Su et al., 2013). High-fat diet-induced obesity in mice caused an increase in ROS and a reduction in AKT and eNOS phosphorylation compared to non-obese mice (Du et al., 2013).

Treatment with cigarette smoke extract (CSE) also inhibited Akt and eNOS in VEGF-stimulated HUVECs (Michaud et al., 2006). Myocardial ischemia decreased phosphorylated AKT and eNOS in spontaneously hypertension (SHR) rats compared to sham animals (Zhang et al., 2014).

Empirical Support for Linkage
Include consideration of temporal concordance here

Treatment with 30 µM methylglyoxal (MG) and 25 mM glucose (HG) for 24 hours caused an increase in ROS (measured by DCF fluorescence in arbitrary units; control: 0.77, MG: 1.2, HG: 1) and a decrease in eNOS activity (control: 100%, MG: 55%, HG: 66%) in HUVECs (Dhar et al., 2010). SIN-1 treatment for two hours increased ROS (100% to 290%) and decreased eNOS activity (100% to 70%) (Das et al., 2014). An increase in ROS and decrease in eNOS activity was observed following treatment with 100 µmol/L H_2O_2 (Chen et al., 2010). Another study showed that 5 µM H_2O_2 treatment initially increased eNOS Ser1179 phosphorylation and activity, but after the peak increase at 30

minutes, eNOS Ser1179 phosphorylation dramatically decreased (Hu et al., 2008). A similar trend was observed for AKT phosphorylation. Treatment with 100 µM/L H_2O_2 for 30 minutes inhibited eNOS expression (Chen et al., 2010), assuming that eNOS expression translates to eNOS activity. Treatment with H_2O_2 (400 µM, 30 min) increased eNOS phosphorylation at the inhibitory site Thr495, suggesting eNOS activity decreased (Guterbaum, 2013).

Uncertainties or Inconsistencies

There are many studies examining the effect of H_2O_2 on AKT/eNOS phosphorylation, but there are conflicting results. Exposure to H_2O_2 for 30 minutes resulted in an increase in AKT/eNOS phosphorylation, but its concentration was much higher at 200 µM (Barbosa et al., 2013) compared to 5 µM in Hu et al. (2008). Another study found that treatment with 50 µM H_2O_2 increased eNOS phosphorylation at Ser1177 (Kumar et al., 2010). Results from studies with H_2O_2 as a source of ROS may not be universally applicable to this key event relationship.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Dhar et al. (2010) showed that an increase in ROS of >30% due to high glucose and methylglyoxal treatment was able to decrease eNOS activity, while Das et al. (2014) showed a three-fold increase in ROS led to a 30% reduction in eNOS activity. SIN-1, high glucose, methylglyoxal and H_2O_2 were demonstrated to modulate both key events at the same time.

References

Barbosa, V.A., Luciano, T.F., Marques, S.O., Vitto, M.F., Souza, D.R., Silva, L.A., Santos, J.P.A., Moreira, J.C., Dal-Pizzol, F., Lira, F.S., et al. (2013). Acute exercise induce endothelial nitric oxide synthase phosphorylation via Akt and AMP-activated protein kinase in aorta of rats: Role of reactive oxygen species. Int. J. Cardiol. 167, 2983–2988.

Chen, X., Xu, J., Feng, Z., Fan, M., Han, J., and Yang, Z. (2010). Simvastatin combined with nifedipine enhances endothelial cell protection by inhibiting ROS generation and activating Akt phosphorylation. Acta Pharmacol. Sin. 31, 813–820.

Dhar, A., Dhar, I., Desai, K.M., and Wu, L. (2010). Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. Br. J. Pharmacol. 161, 1843–1856.

Das, A., Gopalakrishnan, B., Druhan, L.J., Wang, T.-Y., De Pascali, F., Rockenbauer, A., Racoma, I., Varadharaj, S., Zweier, J.L., Cardounel, A.J., et al. (2014). Reversal of SIN-1-induced eNOS dysfunction by the spin trap, DMPO, in bovine aortic endothelial cells via eNOS phosphorylation. Br. J. Pharmacol. 171, 2321–2334.

Du, J., Fan, L.M., Mai, A., and Li, J.-M. (2013a). Crucial roles of Nox2-derived oxidative stress in deteriorating the function of insulin receptors and endothelium in dietary obesity of middle-aged mice. Br. J. Pharmacol. 170, 1064–1077.

Du, X.L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., and Brownlee, M. (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J. Clin. Invest. 108, 1341–1348.

Guterbaum, T.J., Braunstein, T.H., Fossum, A., Holstein-Rathlou, N.-H., Torp-Pedersen, C.T., and Domínguez, H. (2013). Endothelial nitric oxide synthase phosphorylation at Threonine 495 and mitochondrial reactive oxygen species formation in response to a high H_2O_2 concentration. J. Vasc. Res. 50, 410–420.

Hu, Z., Chen, J., Wei, Q., and Xia, Y. (2008). Bidirectional actions of hydrogen peroxide on endothelial nitric-oxide synthase phosphorylation and function: co-commitment and interplay of Akt and AMPK. J. Biol. Chem. 283, 25256–25263.

Kumar, S., Sud, N., Fonseca, F.V., Hou, Y., and Black, S.M. (2010). Shear stress stimulates nitric oxide signaling in pulmonary arterial endothelial cells via a reduction in catalase activity: role of protein kinase C delta. Am. J. Physiol. Lung Cell. Mol. Physiol. 298, L105–L116.

Michaud, S.E., Dussault, S., Groleau, J., Haddad, P., and Rivard, A. (2006). Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species. J. Mol. Cell. Cardiol. 41, 275–284.

Song, P., Wu, Y., Xu, J., Xie, Z., Dong, Y., Zhang, M., and Zou, M.-H. (2007). Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. Circulation 116, 1585–1595.

Song, P., Xie, Z., Wu, Y., Xu, J., Dong, Y., and Zou, M.-H. (2008). Protein kinase Czeta-dependent LKB1 serine 428 phosphorylation increases LKB1 nucleus export and apoptosis in endothelial cells. J. Biol. Chem. 283, 12446–12455.

Su, Y., Qadri, S.M., Wu, L., and Liu, L. (2013). Methylglyoxal modulates endothelial nitric oxide synthase-associated functions in EA.hy926 endothelial cells. Cardiovasc. Diabetol. 12, 134.

Zhang, W., Han, Y., Meng, G., Bai, W., Xie, L., Lu, H., Shao, Y., Wei, L., Pan, S., Zhou, S., et al. (2014). Direct renin inhibition with aliskiren protects against myocardial ischemia/reperfusion injury by activating nitric oxide synthase signaling in spontaneously hypertensive rats. J. Am. Heart Assoc. 3, e000606.

Zou, M.-H., Hou, X.-Y., Shi, C.-M., Nagata, D., Walsh, K., and Cohen, R.A. (2002). Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J. Biol. Chem. 277, 32552–32557.

Peptide Oxidation leads to S-Glutathionylation, eNOS (https://aopwiki.org/relationships/1698)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Peptide Oxidation Leading to Hypertension (https://aopwiki.org/aops/149)	**directly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cow	Bos taurus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)

Life Stage Applicability

Life Stage	Evidence
All life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The above evidence demonstrates similar responses to stressors in cows, mice and humans. A functional response using aortic rings was demonstrated in rats.

How Does This Key Event Relationship Work

Oxidation of GSH results in the formation of a disulphide-bridged glutathione dimer (GSSG). GSSG is either rapidly re-reduced back to GSH by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent GSSG-reductases or extruded from the cell by adenosine triphosphate (ATP)-dependent translocases. However, when these mechanisms become overwhelmed by high local oxidant concentrations, GSSG can interact with protein thiol groups to form protein-GSSG adducts, a process termed S-glutathionylation. Interestingly, glutathione disulfide-protein formation has been suggested to occur with a certain degree of specificity to cellular proteins, since protein thiol groups exhibit a considerable heterogeneity in terms of their individual pK_a values and their location in protein structures (Schuppe et al. 1992). The oxidation of GSH to GSSG elevates levels of GSSG, which then covalently bind to critical serine residues on endothelial nitric oxide synthase (eNOS; Chen et al 2010, Du et al. 2013, De Pascali et al. 2014).

Weight of Evidence

Biological Plausibility
When antioxidant defence mechanisms become overwhelmed by high local oxidant concentrations, GSSG can interact with protein thiol groups to form protein-GSSG adducts, a process termed S-glutathionylation (Schuppe et al. 1992).

Hypoxia/reoxygenation-induced oxidative stress (associated with ischaemia-reperfusion injury) was shown to deplete GSH in bovine aortic endothelial cells, which led to S-glutathionylation of eNOS and eNOS uncoupling. This phenomenon was partially reversible, in bovine aortic endothelial cells and rat aortic rings, by raising intracellular GSH levels upon administration of N-acetylcysteine (Chen et al. 2010, DePascali et al. 2014).

Empirical Support for Linkage
Chen and colleagues (2010) have shown that eNOS is particularly sensitive to S-glutathionylation at cysteine residues 689 and 908 of the reductase domain, a phenomenon that is dose-dependent with application of exogenous GSSG. This finding was corroborated by Peng *et al.* (2015) using mutated eNOS constructs in *E. coli*, demonstrating that superoxide was produced by the eNOS phosphorylation site in the reductase domain.

Wu *et al.* (2014) studied responses in human lung microvascular endothelial cells to lipopolysaccharide (LPS) *in vitro*. Upon LPS administration, NADPH oxidase 2 (NOX2) expression levels were increased with a subsequent rise in superoxide production, which led to S-glutathionylation of eNOS. Furthermore, in mice, co-immunoprecipitation studies revealed that NOX2 associated with eNOS, and that S-glutathionylation in response to LPS was much more apparent in elderly animals compared to younger animals. Similar observations were made by De Pascali *et al.* (2014) following hypoxia-induced oxidative stress in bovine aortic endothelial cells.

Uncertainties or Inconsistencies
Quantitative data for humans is very limited.

Quantitative Understanding of the Linkage
Chen *et al.* (2013) demonstrated that co-administration of glutaredoxin-1 and GSH reversed GSSG-mediated eNOS S-glutathionylation and restored eNOS-mediated NO production, also in bovine aortic cells. Interestingly, inhibition of eNOS function occurred when the GSH/GSSG ratio was >0.2 and function was restored at a ratio of <0.1.

References

Chen CA, Wang TY, Varadharaj, S *et al.* S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature (2010) 468: 1115–1118.

Chen CA, De Pascali F, Basye A *et al.* Redox modulation of endothelial nitric oxide synthase by glutaredoxin-1 through reversible oxidative post-translational modification. (2013) Biochemistry. 52(38):6712-23

Dhar A, Dhar I, Desai KM *et al.* Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. (2010) Br. J. Pharmacol. 161: 1843–1856.

De Pascali F, Hemann C, Samons, K *et al.* Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (2014). 53 : 3679–3688.

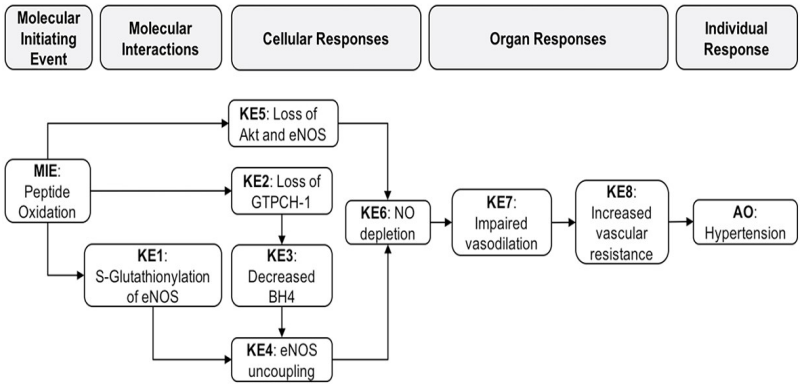
Du Y, Navab M, Shen M, *et al.* Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. (2013) 436 : 462–466.

Peng H, Zhuang Y, Chen Y *et al.* The Characteristics and Regulatory Mechanisms of Superoxide Generation from eNOS Reductase Domain. (2015) PLoS One. 10(10):e0140365

Schuppe I, Moldéus P, and Cotgreave IA. Protein-specific S-thiolation in human endothelial cells during oxidative stress. (1992) Biochem. Pharmacol. 44: 1757–1764.

Wu F, Szczepaniak WS, Shiva S *et al.* Nox2-dependent glutathionylation of endothelial NOS leads to uncoupled superoxide production and endothelial barrier dysfunction in acute lung injury. (2014) Am J Physiol Lung Cell Mol Physiol. 307(12):L987-97.

Graphical Representation



Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
cow	Bos taurus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9913)

Sex Applicability

Sex	Evidence
Unspecific	

Life Stage Applicability, Taxonomic Applicability, Sex Applicability
Elaborate on the domains of applicability listed in the summary section above. Specifically, provide the literature supporting, or excluding, certain domains.

This proposed AOP is applicable to males and females, however a single study did suggest that KE responses may vary with sex (Scotland et al. 2005).

The AOP is relevant for all life stages, as vascular oxidative stress (a common cause of the MIE) can occur at all life stages. However, the AO via this mechanism is likely limited to adults, due to a loss of vaso-protective mechanisms with age (a major risk factor) and cardiovascular re-modelling over time. Hypertension is known to occur in adolescents and children, however the condition can be due to a variety of causes, such as renal disease and cardiovascular complications in early life. For example, persistant pulmonary hypertension is a consequence of failed pulmonary vascular transition at birth and leads to pulmonary hypertension with shunting of deoxygenated blood across the ductus arteriosus and foramen ovale, resulting in severe hypoxemia. The condition is improved by administration of nitric oxide by inhalation (Lai et al. 2017). While the initial cause of the hypertension is not vascular oxidative stress, ROS-mediated perturbations of the vascular endothelium, which arise as a result of the hypoxic conditions, contribute to the development of hypertension, and may have health implications for the neonate later in life (de Wijs-Meijler et al. 2017). Therefore, the physiology described in the AOP is relevant to the condition, but not strictly causative.

The KEs in this AOP are well-documented and well-studied in humans, cows and rodents. Similar outcomes are observed across these species following chemical exposure and other phenomena which cause vascular oxidative stress (e.g. hypoxia, ischaemia/reperfusion).

The specific amino-acid residues involved in post-translational modification of eNOS do differ across species, however the functional effect is similar. For example, human eNOS is phosphorylated by AKT at serine residue 1177 (Reviewed by Heiss et al. 2014), whereas bovine eNOS is phosphoryated by AKT at serine 1179 (Chen et al. 2017).

Essentiality of the Key Events

Molecular Initiating Event Summary, Key Event Summary
Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

The essentiality for this AOP is strong since there is direct evidence from multiple experimental studies showing that downstream key events can be prevented or inhibited if an upstream key event is blocked. An increase in intracellular glutathione, rather than the oxidized form, prevented S-glutathionylation (De Pascali et al., 2014), and inhibiting S-glutathionylation attenuated ultrafine particle-induced reduction in NO production (Du et al., 2013). In addition, increasing GTPCH-1, BH4 and Akt/eNOS activity increased

eNOS activity and NO production but decreased superoxide generation (Fulton et al., 1999; Alp et al., 2003; Carnicer et al., 2012; Antoniades et al., 2011; Chen et al., 2011; De Pascali et al., 2014; Landmessar et al., 2003; Shinozaki et al., 2000; Ozaki et al., 2002). Infusion of NO donor sodium nitroprusside reduced vascular resistance, while sodium nitrite increased vasodilation (Eugene, 2016; Sindlier et al., 2014).

The essentiality of increased vascular resistance to hypertension is moderate due to the involvement of the heart in regulating blood pressure. While increased vascular resistance is a hallmark of essential hypertension, Mayet and Hughes (2003) discussed the roles of the heart and vascular resistance and concluded that both are of importance, and should not be viewed in isolation. Their conclusion is drawn from observations in younger adults with elevated blood pressure, who have a normal vascular resistance, but higher cardiac index. Over time, such adults often go on to change to a phenotype with a lower cardiac index and high vascular resistance and a diagnosis of chronic hypertension. Such changes are likely due to vascular remodelling processes with advancing age, however the specifics of this process are not well understood.

Support for Essentiality of KEs	KE Description	Defining Question Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	High Direct evidence from experimental studies illustrating essentiality for at least one of the important KEs.	Moderate Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE.	Low No or contradictory experimental evidence of the essentiality of any of the KEs.
Peptide Oxidation (MIE)	Generation of localised ROS and other oxidative species in the vascular endothelium leads to oxidation of amino acid residues of peptides.				
S-glutathionylation of eNOS (KE1)	The oxidation of GSH into GSSG, elevates levels of GSSG, which then covalently bind to critical serine residues on the eNOS enzyme	High	Protein S-Glutathionylation can only occur following covalent binding of GSSG. GSSG commonly binds to protein thiol residues, which can affect protein function (Bendall et al 2014). S-glutathionylation of eNOS is observed under conditions of oxidative stress, at cysteine residues 689 and 908 leading to eNOS uncoupling (Chen et al. 2010). Application of Glutaredoxin-1 or dithiothreitol removes S-glutathionylation and restores eNOS function (Chen et al. 2013, Jayaram et al. 2015) Essentiality rating is high due to direct experimental evidence showing removal of S-glutathionylation of eNOS restores NO production.		
Loss of GTPCH-1 (KE2)	Oxidative damage to GTPCH-1 has been shown to lead to reduced expression/activity of the enzyme	High	Numerous studies have demonstrated that deletion of GTPCH-1 led to the deficiency of BH4 in bovine and murine endothelial cells (Tatham et al. 2009, Crabtree et al. 2009, Adlam et al. 2012) and in knockout mice (Chen et al. 2011). Gene silencing and GTPCH-1 inhibition lead to elevated blood pressure in experimental animals (Wang et al. 2008, Mitchell et al. 2003). Essentiality rating is high due to sunstantial experimental evidence of GTPCH-1 blocking on downstream KEs.		
Decreased BH4 (KE3)	Oxidation of BH4 itself to BH2, and/or reduced bioavailability of BH4 by a reduction in de novo synthesis by GTPCH-1.	High	Depletion of BH4 has been extensively reported to uncouple eNOS, decrease NO bioavailability and increase superoxide production. Application of exogenous BH4 has been reported to reverse this phenomenon (De Pascali et al. 2014, Zweier et al. 2011, Dumitrescu et al. 2007, Tiefenbacher et al. 1996, Vásquez-Vivar et al. 2002) Essentiality rating is high due to extensive direct experimental evidence.		
eNOS Uncoupling (KE4)	eNOS uncoupling is characterised by a loss of NO production and superoxide production as eNOS dimerisation is lost	High	eNOS uncoupling leads to reduction in NO bioavailability. Reversal of eNOS uncoupling is achieved by supplementation with BH4 or removal of eNOS S-glutathionylation (as described earlier), which restores NO production (Du et al. 2013b, Nozik-Grayck et al. 2014, Talukder et al. 2011, Su et al. 2013). Essentiality rating is high due to direct experimental evidence.		
Loss of AKT/eNOS (KE5)	In humans, AKT phosphorylates eNOS at serine residue 1177, leading to eNOS activation and NO production. Loss of AKT/eNOS function depletes NO bioavailability.	High	eNOS knockout mice are routinely used as models of hypertension (Huang 2000). Aortic rings from eNOS knockout mice do not relax following application of acetylcholine, but do relax upon application of NO donor sodium nitroprusside (Huang et al. 1995). Many animal studies demonstrated that inhibition of NO via eNOS inhibitors impaired endothelium-dependent vasodilation (Li et al. 2007, Paulis et al. 2008, Luo et al. 2000, Sélley et al. 2014). Inhibition of Akt or mutant eNOS attenuated eNOS phosphorylation in human and bovine cells, resulting in decreased NO bioavailability (Michaud et al. 2006, Dhar et al. 2010, Dimmeler et al. 1999, Fulton et al. 1999, Das et al. 2014, Uruno et al. 2005). Essentiality rating is high due to extensive direct experimental evidence.		
NO Depletion (KE6)	Nitric oxide is a potent vasodilator radical released by endothelial (cell) eNOS. NO signalling leads to potassium ion efflux and hyperpolarization of vascular smooth muscle cells and blood vessel relaxation. Reduction in NO bioavailability blunts this response	Moderate	Depletion of NO bioavailability by pharmacological blockade of AKT and eNOS is widely reported to shift vascular tone towards a more vasoconstrictive phenotype, leading to hypertension (Li et al. 2007, Paulis et al. 2008, Scotland et al. 2005, Luo et al. 2000, Sélley et al. 2014, Haynes et al. 1993). However gender differences in experimental animals were reported to influence vasodilation by different mechanisms (Scotland et al. 2005). Furthermore, compensatory mechanisms affecting vascular tone are evident when bioavailability of NO is decreased (Brandes 2014, Durand et al. 2013). Essentiality rating is moderate due to extensive evidence from pharmacological blocking studies in humans and animals which show impairment of vasodilation and elevated BP. However, compensatory effects of other biological mechanisms on downstream KEs are evident as are gender differences in experimental animals.		

Impaired vasodilation (KE7)	Vasodilation is mediated by vascular smooth muscle cells and characterized by potassium ion efflux and hyperpolarization of the tissue in response to signalling from the nervous system and chemical mediators released by the vascular endothelium	Moderate	It is well accepted that vasodilation and systemic vascular resistance (SVR) are negatively correlated. Blood flow volume is increased when blood vessels dilate due to decreased vascular resistance (Siddiqui 2011). When vasodilation is impaired as a result of NO depletion or blockade of potassium channels, vascular stiffness and SVR increase (Berg et al. 2011, Brett et al. 1998, Dessey et al. 2004, Li et al. 2007, McVeigh et al. 2001, Paulis et al. 2008, Wilkinson et al. 2002). Essentiality rating is high due to extensive direct experimental evidence.
Increased vascular resistance (KE8)	Increases in vascular tone as a result of impaired vasodilation. Commonly referred to as systemic vascular resistance (SVR) or total peripheral resistance (TPR).	Moderate	It is well established that increased SVR (TPR), increased vascular stiffness and increased vascular reactivity contribute to hypertension (Brandes 2014, Foëx and Sear 2004, Mayet and Hughes 2003). In patients with hypertension, SVR was elevated in approximately 66% of enrolled patients (Chan et al. 2016). However, due to the critical role of cardiac output in blood pressure regulation (Brandes 2014, Mayet and Hughes 2003), and other compensatory mechanisms described earlier, the essentiality for the AO is moderate.
Hypertension (AO)	Chronic elevation of systolic and/or diastolic blood pressure in systemic circulation or localised organs		

Weight of Evidence Summary

Summary Table

Provide an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages.

Quantitative measurements with dose and time response data from published studies cited below can be found here: File:Hypertension Empirical Support Concordance Table.pdf (https://aopwiki.org/wiki/index.php/File:Hypertension_Empirical_Support_Concordance_Table.pdf).

Support for Biological Plausibility of KERs	**Defining Question**	**High (Strong)**	**Moderate**	**Low (Weak)**
	Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on previous documentation and broad acceptance.	KER is plausible based on analogy to,accepted biological relationships, but scientific understanding is incomplete.	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
Oxidative Stress, Increase Directly Leads to Glutathione, Oxidation:	Strong	Multiple studies demonstrated that oxidative stress leads to the oxidation of glutathione (GSH) in the vascular endothelium. Exposure to a number of oxidants, including tert-butyl hydroperoxide, hydrogen peroxide, diamide, methylglyoxal, glucose, ischemia, and ultrafine particles caused a decrease in levels of GSH, which is indicative of its oxidation in human, bovine, and rat endothelial cells (De Pascali et al., 2014; Dhar et al., 2010; Du et al., 2013b; Montecinos et al., 2007; Park, 2013; Schuppe et al., 1992; van Gorp et al., 1999, 2002).		
Oxidative Stress, Increase Directly Leads to AKT/eNOS activity, Decrease	Strong	Multiple experimental studies reported decreased Akt and eNOS phosphorylation/activity following oxidative stress as a consequence of exposure to peroxynitrite, high glucose, methylglyoxal, high fat, cigarette smoke extract (CSE) and ischemia in humans, bovine, mouse and rat endothelial cells (Das et al., 2014; Dhar et al., 2010; Du et al., 2001; Du et al., 2013; Michaud et al., 2006; Song et al., 2007, 2008; Su et al., 2013; Zhang et al., 2014; Zou et al., 2002).		
AKT/eNOS activity, Decrease Directly Leads to Nitric Oxide, Depletion	Strong	Several studies demonstrated that Akt can directly phosphorylate eNOS, leading to increased eNOS enzymatic activity and subsequent NO production (Dimmeler et al., 1999; Fulton et al., 1999). Inhibition of Akt or mutant eNOS attenuated eNOS phosphorylation in human and bovine cells, resulting in decreased NO bioavailability (Das et al., 2014; Dhar et al. 2010; Dimmeler et al., 1999; Fulton et al., 1999; Michaud et al., 2006; Uruno et al., 2005).		
Oxidative Stress, Increase Directly Leads to GTPCH-1, Decrease	Moderate	Several studies demonstrated that GTPCH-1, the rate-limiting enzyme for BH4 synthesis, is affected by oxidative stress. GTPCH-1 expression or activity was inhibited by peroxynitrite and CSE (Abdelghany et al., 2017; Zhao et al., 2003). Cardiac reperfusion patients who experienced oxidative stress had reduced GTPCH-1 activity (Jayaram et al., 2015).		
GTPCH-1, Decrease Directly Leads to Tetrahydrobiopterin, Decrease	Strong	Many studies demonstrated that GTPCH-1 deletion led to reduced bioavailability of BH4 in bovine and murine endothelial cells (Adlam et al., 2012; Chen et al., 2011; Chuaiphichai et al., 2014; Crabtree et al., 2009; Tatham et al., 2009; Wang et al., 2008). Several studies also showed that overexpression of GTPCH-1 in human and mouse endothelium increased BH4 levels and eNOS activity (Alp et al., 2003; Antoniades et al., 2011; Carnicer et al., 2012).		

Tetrahydrobiopterin, Decrease Directly Leads to eNOS, Uncoupling	Strong	The depletion of BH4 leading to eNOS uncoupling is well-studied. Several studies showed reduced levels of BH4 induced eNOS uncoupling by reducing eNOS activity, decreasing NO and increasing superoxide levels in bovine, murine, and rat endothelium (Chuaiphichai et al., 2014; Crabtree et al., 2009; De Pascali et al., 2014; Dumitrescu et al., 2007; Whitsett et al., 2007). Many studies demonstrated that BH4 treatment improved endothelial function by reducing eNOS-mediated superoxide generation and increasing NO formation in human, bovine, mouse, and rat endothelium (Chen et al., 2011; De Pascali et al., 2014; Landmesser et al., 2003; Ozaki et al., 2002; Shinozaki et al., 2000; Wang et al., 2014).
Glutathione, Oxidation Directly Leads to eNOS, S-Glutathionylation	Strong	Glutathione oxidation as determined by increased oxidized GSSG or decreased GSH levels caused S-glutathionylation of eNOS in bovine and human aortic endothelial cells, and in hypertensive rats and mice (Chen et al., 2010; De Pascali et al., 2014; Du et al., 2013).
eNOS, S-Glutathionylation Directly Leads to eNOS, Uncoupling	Strong	*In vitro* experiments showed that S-glutathionylation of eNOS significantly decreased NO activity and greatly increased superoxide generation (Chen et al., 2010). These results were observed in bovine and human aortic endothelial cells as well as in vessels of spontaneously hypertensive rats and cardiac reperfusion patients (De Pascali et al., 2014; Du et al., 2013; Jayaram et al., 2015).
eNOS, Uncoupling Directly Leads to Nitric Oxide, Depletion	Strong	It is well-established that uncoupling of eNOS causes eNOS to switch from producing NO to generating superoxides (Förstermann and Münzel, 2006). Studies reporting eNOS uncoupling as a result of BH4 depletion or S-glutathionylation measured levels of NO and superoxide which are indicative of eNOS uncoupling (Chen et al. 2010; De Pascali et al., 2014, Du et al., 2013; Whitsett et al., 2007).
Nitric Oxide, Depletion Directly Leads to Vasodilation, impaired	Strong	Vasodilation is caused by the relaxation of vascular smooth muscle cells within the walls of blood vessels, and is regulated through a number of mechanisms, including cyclic GMP-dependent hyperpolarization and relaxation via NO. Thus, alterations to NO levels have an influence on vasodilation (Silva et al., 2012). Many animal studies demonstrated that inhibition of NO via eNOS inhibitors impaired endothelium-dependent vasodilation (Li et al., 2007; Luo et al., 2000; Paulis et al., 2008; Sélley et al., 2014).
Vasodilation, impaired Directly Leads to Vascular resistance, Increase	Strong	It is well-accepted that vasodilation and systemic vascular resistance (SVR) are negatively correlated; blood flow is increased when blood vessels dilate due to decreased vascular resistance (Siddiqui, 2011). When vasodilation is impaired as a result of NO depletion or changes in potassium channels, vascular tone and SVR increase (Berg and Jensen, 2011; Brett et al., 1998; Dessy et al., 2004; Li et al., 2007; McVeigh et al., 2001; Paulis et al., 2008; Wilkinson et al., 2002).
Vascular resistance, Increase Directly Leads to Hypertension, N/A	Strong	It is well-established that increased systemic vascular resistance (SVR), increased vascular stiffness and increased vascular reactivity contribute to hypertension (Brandes, 2014; Foëx and Sear, 2004; Mayet and Hughes, 2003). In patients with hypertension, SVR was elevated in about 66% of enrolled patients (Chan et al., 2016).

Empirical Support for KERs	**Defining Question**	**High (Strong)**	**Moderate**	**Low (Weak)**
	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses, earlier time points, and higher in incidence than KEdown ? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species
Oxidative Stress, Increase Directly Leads to Glutathione, Oxidation:	Moderate	Many studies demonstrated a dose-dependent relationship between known inducers of oxidative stress (tert-butyl hydroperoxide, hydrogen peroxide, methylglyoxal, high glucose, and ultrafine particles) and reduced GSH levels in human and rat studies (Dhar et al., 2010; Du et al., 2013; Montecinos et al., 2007; Park et al., 2013; van Gorp et al., 1999).		
Oxidative Stress, Increase Directly Leads to AKT/eNOS activity, Decrease	Moderate	eNOS activity and reactive oxygen species (ROS) were modulated in the opposite manner by several stressors (methylglyoxal, high glucose, SIN-1, hydrogen peroxide) in human and bovine endothelial cells, resulting in increased ROS and decreased eNOS activity (Das et al., 2014; Dhar et al., 2010; Chen et al., 2010).		
AKT/eNOS activity, Decrease Directly Leads to Nitric Oxide, Depletion	Strong	Various stress inducers (ischemia, peroxynitrite, SIN-1, insulin plus orotic acidura, etc.) showed that a decrease in AKT and/or eNOS activity led to increased eNOS uncoupling and decreased NO (Choi et al., 2014, 2015; Das et al., 2014; Dhar et al., 2010; Dumitrescu et al., 2007 Uruno et al., 2005).		
Oxidative Stress, Increase Directly Leads to GTPCH-1, Decrease	Weak	One study in a rat model of aortic coarctation-associated hypertension provides evidence that there is a interdependence between oxidative stress and GTPCH-1 with increased ROS and decreased GTPCH-1 expression following a perturbation, but there is no dose-response or temporal data (Cervantes-Pérez et al., 2012).		

GTPCH-1, Decrease Directly Leads to Tetrahydrobiopterin, Decrease	Strong	Exposure to a wide range of perturbations (e.g. CSE, 4-hydroxy-2-nonenal, cytokines) led to a decrease in both GTPCH-1 activity/expression and BH4 levels in human, cows and rats (Antoniades et al., 2011; Cervantes-Pérez et al., 2012; Chen et al., 2011; Ismail et al., 2015; Jayaram et al., 2015; Whitsett et al., 2007).
Tetrahydrobiopterin, Decrease Directly Leads to eNOS, Uncoupling	Strong	Multiple studies demonstrated strong dependency between BH4 and eNOS uncoupling; decreased BH4 along with decreased eNOS activity, decreased NO production or increased superoxide generation were observed after various perturbations (Cervantes-Pérez et al., 2012; De Pascali et al., 2014; Dumitrescu et al., 2007; Jayaram et al., 2015; Whitsett et al., 2007; Wang et al., 2008).
Glutathione, Oxidation Directly Leads to eNOS, S-Glutathionylation	Moderate	Treatment with GSSG induced a dose-dependent increase in human eNOS S-glutathionylation *in vitro* whereas 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) treatment also resulted in increased eNOS S-glutathionylation in a dose-dependent manner in bovine aortic endothelial cells (Chen et al., 2010). Exposure to ultrafine particles or hypoxia/reoxygenation in human or bovine aortic endothelial cells respectively, demonstrated a response-response relationship between reduced glutathione (GSH) and eNOS S-glutathionylation (Du et al., 2013; De Pascali et al., 2014).
eNOS, S-Glutathionylation Directly Leads to eNOS, Uncoupling	Moderate	Treatment with BCNU resulted in increased eNOS S-glutathionylation, increased superoxide generation and decreased NO production in a dose-dependent manner in bovine aortic endothelial cells (Chen et al., 2010). Exposure to hypoxia/reoxygenation and treatment with angiotensin II demonstrated a response-response relationship between eNOS S-glutathionylation and superoxide generation in human and bovine endothelial cells (De Pascali et al., 2014; Galougahi et al., 2014).
eNOS, Uncoupling Directly Leads to Nitric Oxide, Depletion	Strong	Multiple experiments demonstrated that eNOS uncoupling results in increased superoxide formation and decreased NO production (Chen et al., 2010; De Pascali et al., 2014; Dumitrescu et al., 2007; Wang et al., 2008; Whitsett et al., 2007; Zou et al., 2002).
Nitric Oxide, Depletion Directly Leads to Vasodilation, impaired	Moderate	Treatment with eNOS inhibitors and two other stressors, BCNU and diaminohydroxypyrimidine (DAHP), caused a decrease in both NO production and vasodilation (Chen et al., 2010; Paulis et al, 2008; Wang et al., 2008).
Vasodilation, impaired Directly Leads to Vascular resistance, Increase	Weak	No direct evidence was found for this KER, but there is indirect support. Treatment with eNOS inhibitor L-NG-monomethyl arginine citrate (L-NMMA) caused an increase in SVR and a reduction in NO (Stamler et al., 1994), while L-NG-nitroarginine methyl ester (L-NAME) decreased NO-dependent relaxation and increased blood pressure (Paulis et al, 2008). Infusion of NO donor sodium nitroprusside led to dose-dependent reductions in SVR (Eugene, 2016).
Vascular resistance, Increase Directly Leads to Hypertension, N/A	Moderate	Several human studies showed a dose-dependent change in SVR and hypertension following treatment with eNOS inhibitors (Brett et al., 1998; Haynes et al., 1993; McVeigh et al., 2001; Stamler et al., 1994; Wilkinson et al., 2002).

Quantitative Consideration

Summary Table

Provide an overall discussion of the quantitative information available for this AOP. Support calls for the individual relationships can be included in the Key Event Relationship table above.

Overall, there is a good amount of quantitative data available for this AOP as demonstrated by the weight of evidence tables, which shows that empirical support ranges from weak to strong. Four key event relationships (KERs) have strong quantitative support: decreased AKT/eNOS activity => NO depletion, decreased GTPCH-1 => decreased BH4, decreased BH4 => eNOS uncoupling and eNOS uncoupling => NO depletion. The relationships between these key events are well described in the literature as GTPCH-1 is the rate-limiting enzyme for BH4 synthesis (Wang et al., 2008), BH4 is an essential cofactor for eNOS and function (Wang et al., 2014), and the uncoupling of eNOS causes it to generate superoxide instead of NO (Carnicer et al., 2012). As they are functionally interconnected, many studies measure these key events together; thus providing strong support for their dependency.

Two key events have limited quantitative support (oxidative stress => decreased GTPCH-1, impaired vasodilation => increased vascular resistance). Generally, the oxidation of BH4 rather than decreased GTPCH-1 is measured when cells are under oxidative stress, and ROS are assumed to be increased so no quantitative measures are taken. For vasodilation and vascular resistance, there appears to be a correlative relationship, where increased vasodilation would mean decreased vascular resistance and vice versa, so studies do not measure both key events. Several studies showed that treatment with eNOS inhibitors led to increased vascular resistance, suggesting impaired vasodilation (Li et al., 2007; McVeigh et al., 2001; Paulis et al., 2008; Wilkinson et al., 2002).

The other KERs have moderate quantitative support, meaning there were studies showing a dependent change in both key events following treatment with stressors. Several studies measured decreased NO and vasodilation following perturbations to eNOS inhibitors, BCNU and DAHP (Chen et al., 2010; Paulis et al, 2008; Wang et al., 2008).

In general, experiments with both dose-dependent and temporal response data following a stressor are not readily available for all KERs as most measurements are generally taken at one time point after a perturbation, or the measurements are for one key event, not both. However, an exhaustive literature search was not performed. An ideal experiment would be to treat cells with three to four stressors at increasing concentrations and measure the key events at different time intervals; thus providing a greater understanding of the temporal and dose-dependent responses between the key events.

Considerations for Potential Applications of the AOP (optional)

One of the most widely reported hypertension risk factors is tobacco smoking. While smoking cessation remains the best way to reduce the harmful effects of tobacco smoking, tobacco harm reduction is being considered by some regulators (e.g. US Food and Drug Administration; FDA) as a complementary strategy to reduce smoking-related disease burden. The US FDA has published guidance on assessing a “modified risk tobacco product” (MRTP), either through demonstration of reduced toxicant exposure or reduction in health risks (FDA 2012). By gaining an understanding of how, and to what extent tobacco smoke initiates biological mechanisms of hypertension, such knowledge could be utilised as a baseline for comparison purposes in toxicological assessments of the risk reduction potential of e-cigarettes.

Given the wide variety of data requirements to support such risk assessments e.g. human exposure studies, behavioural studies, efficacy studies, *in vitro* studies, clinical biomarker studies, pharmacokinetic studies, quality of life studies etc., a data integration framework is required to organise these data types into a comprehensive story that (i) characterises the toxicological problem, (ii) demonstrates the likely outcome of an intervention, and (iii) can be utilised to monitor the performance of any intervention over time. Adverse outcome pathways offer scientists and regulators a way to inform future Integrated Approaches to Testing and Assessment (IATA) for the risk assessment/harm reduction potential of electronic cigarettes and heated tobacco products.

Other applications could include informing risk assessments for long term exposure to airborne pollution, in the context of helping to set acceptable exposure limits in urban environments for specific pollutants e.g. diesel exhaust particles. Furthermore, health supplements which purport to benefit the cardiovascular system in the context of hypertension risk, could also be assessed for efficacy.

References

AbdelGhany, T., Ismail, R., Elmahdy, M., Mansoor F, Zweier J, Lowe, F., and Zweier, JL. (2017). Cigarette Smoke Constituents Cause Endothelial Nitric Oxide Synthase Dysfunction and Uncoupling due to Depletion of Tetrahydrobiopterin with Degradation of GTP Cyclohydrolase. Nitric Oxide (Under review).

Adlam, D., Herring, N., Douglas, G., De Bono, J.P., Li, D., Danson, E.J., Tatham, A., Lu, C.-J., Jennings, K.A., Cragg, S.J., et al. (2012). Regulation of β-adrenergic control of heart rate by GTP-cyclohydrolase 1 (GCH1) and tetrahydrobiopterin. Cardiovasc. Res. 93, 694–701.

Alp, N.J., Mussa, S., Khoo, J., Cai, S., Guzik, T., Jefferson, A., Goh, N., Rockett, K.A., and Channon, K.M. (2003). Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. J. Clin. Invest. 112, 725–735.

Antoniades, C., Cunnington, C., Antonopoulos, A., Neville, M., Margaritis, M., Demosthenous, M., Bendall, J., Hale, A., Cerrato, R., Tousoulis, D., et al. (2011). Induction of vascular GTP-cyclohydrolase I and endogenous tetrahydrobiopterin synthesis protect against inflammation-induced endothelial dysfunction in human atherosclerosis. Circulation 124, 1860–1870.

Bendall JK, Douglas G, McNeill E *et al.* Tetrahydrobiopterin in cardiovascular health and disease. Antioxid Redox Signal. (2014) 20(18):3040-77.

Berg, T., and Jensen, J. (2011). Simultaneous parasympathetic and sympathetic activation reveals altered autonomic control of heart rate, vascular tension, and epinephrine release in anesthetized hypertensive rats. Front. Neurol. 2, 71.

Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem. 1997, 272(33):20313-6.

Bhatnagar, A., Whitsel, L.P., RibisI, K.M., Bullen, C., Chaloupka, F., Piano, M.R., Robertson, R.M., McAuley, T., Goff, D., Benowitz, N., et al. (2014). Electronic cigarettes: a policy statement from the American Heart Association. Circulation 130, 1418–1436.

Brandes, R.P. (2014). Endothelial dysfunction and hypertension. Hypertension 64, 924–928.

Brett, S.E., Cockcroft, J.R., Mant, T.G., Ritter, J.M., and Chowienczyk, P.J. (1998). Haemodynamic effects of inhibition of nitric oxide synthase and of L-arginine at rest and during exercise. J. Hypertens. 16, 429–435.

Carnicer, R., Hale, A.B., Suffredini, S., Liu, X., Reilly, S., Zhang, M.H., Surdo, N.C., Bendall, J.K., Crabtree, M.J., Lim, G.B.S., et al. (2012). Cardiomyocyte GTP cyclohydrolase 1 and tetrahydrobiopterin increase NOS1 activity and accelerate myocardial relaxation. Circ. Res. 111, 718–727.

Cervantes-Pérez, L.G., Ibarra-Lara, M. de la L., Escalante, B., Del Valle-Mondragón, L., Vargas-Robles, H., Pérez-Severiano, F., Pastelín, G., and Sánchez-Mendoza, M.A. (2012). Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension. Eur. J. Pharmacol. 685, 108–115.

Chan, S.S., Tse, M.M., Chan, C.P., Tai, M.C., Graham, C.A., and Rainer, T.H. (2016). Haemodynamic changes in emergency department patients with poorly controlled hypertension. Hong Kong Med. J. Xianggang Yi Xue Za Zhi Hong Kong Acad. Med. 22, 116–123.

Chen, C.-A., Wang, T.-Y., Varadharaj, S., Reyes, L.A., Hemann, C., Talukder, M.A.H., Chen, Y.-R., Druhan, L.J., and Zweier, J.L. (2010a). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 468, 1115–1118.

Chen, C.-A., Lin, C.-H., Druhan, L.J., Wang, T.-Y., Chen, Y.-R., and Zweier, J.L. (2011). Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. J. Biol. Chem. 286, 29098–29107.

Chen CA, De Pascali F, Basye A *et al.* Redox modulation of endothelial nitric oxide synthase by glutaredoxin-1 through reversible oxidative post-translational modification. (2013) Biochemistry. 52(38):6712-23

Chen Y, Jiang B, Zhuang Y, Peng H, Chen W. Differential effects of heat shock protein 90 and serine 1179 phosphorylation on endothelial nitric oxide synthase activity and on its cofactors. PLoS One. 2017 12(6):e0179978.

Choi, Y.-J., Yoon, Y., Lee, K.-Y., Hien, T.T., Kang, K.W., Kim, K.-C., Lee, J., Lee, M.-Y., Lee, S.M., Kang, D.-H., et al. (2014). Uric acid induces endothelial dysfunction by vascular insulin resistance associated with the impairment of nitric oxide synthesis. FASEB J. 28, 3197–3204.

Choi, Y.-J., Yoon, Y., Lee, K.-Y., Kang, Y.-P., Lim, D.K., Kwon, S.W., Kang, K.-W., Lee, S.-M., and Lee, B.-H. (2015). Orotic Acid Induces Hypertension Associated with Impaired Endothelial Nitric Oxide Synthesis. Toxicol. Sci. 144, 307–317.

Chuaiphichai, S., McNeill, E., Douglas, G., Crabtree, M.J., Bendall, J.K., Hale, A.B., Alp, N.J., and Channon, K.M. (2014). Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 64, 530–540.

Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., and Alp, N.J. (2009). Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1136–1144.

Das, A., Gopalakrishnan, B., Druhan, L.J., Wang, T.-Y., De Pascali, F., Rockenbauer, A., Racoma, I., Varadharaj, S., Zweier, J.L., Cardounel, A.J., et al. (2014). Reversal of SIN-1-induced eNOS dysfunction by the spin trap, DMPO, in bovine aortic endothelial cells via eNOS phosphorylation. Br. J. Pharmacol. 171, 2321–2334.

De Pascali, F., Hemann, C., Samons, K., Chen, C.-A., and Zweier, J.L. (2014). Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. Biochemistry (Mosc.) 53, 3679–3688.

Dessy, C., Moniotte, S., Ghisdal, P., Havaux, X., Noirhomme, P., and Balligand, J.L. (2004). Endothelial beta3-adrenoceptors mediate vasorelaxation of human coronary microarteries through nitric oxide and endothelium-dependent hyperpolarization. Circulation 110, 948–954.

Dhar, A., Dhar, I., Desai, K.M., and Wu, L. (2010). Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. Br. J. Pharmacol. 161, 1843–1856.

Dharmashankar, K., and Widlansky, M.E. (2010). Vascular endothelial function and hypertension: insights and directions. Curr. Hypertens. Rep. 12, 448–455.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

Du, J., Fan, L.M., Mai, A., and Li, J.-M. (2013). Crucial roles of Nox2-derived oxidative stress in deteriorating the function of insulin receptors and endothelium in dietary obesity of middle-aged mice. Br. J. Pharmacol. 170, 1064–1077.

Du, X.L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., and Brownlee, M. (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J. Clin. Invest. 108, 1341–1348.

Du, Y., Navab, M., Shen, M., Hill, J., Pakbin, P., Sioutas, C., Hsiai, T.K., and Li, R. (2013b). Ambient ultrafine particles reduce endothelial nitric oxide production via S-glutathionylation of eNOS. Biochem. Biophys. Res. Commun. 436, 462–466.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A.J., Druhan, L.J., Ambrosio, G., and Zweier, J.L. (2007). Myocardial ischemia results in tetrahydrobiopterin (BH4) oxidation with impaired endothelial function ameliorated by BH4. Proc. Natl. Acad. Sci. U. S. A. 104, 15081–15086.

Durand MJ, Gutterman DD. Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. Microcirculation. (2013) 20(3):239-47

Eugene, A.R. (2016). The influences of nitric oxide, epinephrine, and dopamine on vascular tone: dose-response modeling and simulations. Hosp. Chron. Nosokomeiaka Chron. 11, 1–8.

Food and Drug Administration. Guidance for Industry Modified Risk Tobacco Product Applications. Draft Guidance. Silver Spring, MD, USA: Center for Tobacco Products (2012). http://www.fda.gov/downloads/TobaccoProducts/GuidanceComplianceRegulatoryInformation/UCM297751.pdf (accessed 26 August 2015).

Förstermann, U., and Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.

Foëx, P., and Sear, J.W. (2004). Hypertension: pathophysiology and treatment. Contin. Educ. Anaesth. Crit. Care Pain 4, 71–75.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., and Sessa, W.C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597–601.

Galougahi, K.K., Liu, C.-C., Gentile, C., Kok, C., Nunez, A., Garcia, A., Fry, N.A.S., Davies, M.J., Hawkins, C.L., Rasmussen, H.H., et al. (2014). Glutathionylation Mediates Angiotensin II–Induced eNOS Uncoupling, Amplifying NADPH Oxidase-Dependent Endothelial Dysfunction. J. Am. Heart Assoc. 3, e000731.

van Gorp, R.M.A., Heeneman, S., Broers, J.L.V., Bronnenberg, N.M.H.J., van Dam-Mieras, M.C.E., and Heemskerk, J.W.M. (2002). Glutathione oxidation in calcium- and p38 MAPK-dependent membrane blebbing of endothelial cells. Biochim. Biophys. Acta 1591, 129–138.

van Gorp, R.M., Broers, J.L., Reutelingsperger, C.P., Bronnenberg, N.M., Hornstra, G., van Dam-Mieras, M.C., and Heemskerk, J.W. (1999). Peroxide-induced membrane blebbing in endothelial cells associated with glutathione oxidation but not apoptosis. Am. J. Physiol. 277, C20–C28.

Haddad, P., Dussault, S., Groleau, J., Turgeon, J., Maingrette, F., and Rivard, A. (2011). Nox2-derived reactive oxygen species contribute to hypercholesterolemia-induced inhibition of neovascularization: effects on endothelial progenitor cells and mature endothelial cells. Atherosclerosis 217, 340–349.

Heiss EH, Dirsch VM. Regulation of eNOS Enzyme Activity by Posttranslational Modification. Current pharmaceutical design. 2014;20(22):3503-3513.

Higashi, Y., Maruhashi, T., Noma, K., and Kihara, Y. (2014). Oxidative stress and endothelial dysfunction: clinical evidence and therapeutic implications. Trends Cardiovasc. Med. 24, 165–169.

Huang PL. Mouse models of nitric oxide synthase deficiency. (2000) J Am Soc Nephrol. 11 Suppl 16:S120-3.

Huang PL, Huang Z, Mashimo H *et al.* Hypertension in mice lacking the gene for endothelial nitric oxide synthase. (1995) Nature. 377(6546):239-42.

Jayaram, R., Goodfellow, N., Zhang, M.H., Reilly, S., Crabtree, M., De Silva, R., Sayeed, R., and Casadei, B. (2015). Molecular mechanisms of myocardial nitroso-redox imbalance during on-pump cardiac surgery. Lancet Lond. Engl. 385 Suppl 1, S49.

Lai M, Chu S, Lakshminrusimha S, Lin H. Beyond the inhaled nitric oxide in persistent pulmonary hypertension of the newborn. Pediatr Neonatol. 2017 pii: S1875-9572(17)30480-1. doi: 10.1016/j.pedneo.2016.09.011. [Epub ahead of print]

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E., and Harrison, D.G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest. 111, 1201–1209.

Lee, M.Y., and Griendling, K.K. (2008). Redox signaling, vascular function, and hypertension. Antioxid. Redox Signal. 10, 1045–1059.

Lerman, L.O., Nath, K.A., Rodriguez-Porcel, M., Krier, J.D., Schwartz, R.S., Napoli, C., and Romero, J.C. (2001). Increased oxidative stress in experimental renovascular hypertension. Hypertension 37, 541–546.

Li, J., Zhou, Z., Jiang, D.-J., Li, D., Tan, B., Liu, H., and Li, Y.-J. (2007). Reduction of NO- and EDHF-mediated vasodilatation in hypertension: role of asymmetric dimethylarginine. Clin. Exp. Hypertens. N. Y. N 1993 29, 489–501.

Lowe FJ., Luettich K, Talikka M, Hoang V, Haswell LE., Hoeng J, and Gaca MD. Development of an Adverse Outcome Pathway for the Onset of Hypertension by Oxidative Stress-Mediated Perturbation of Endothelial Nitric Oxide Bioavailability. Applied In Vitro Toxicology. (2017), 3(1): 131-148.

Luo, Z., Fujio, Y., Kureishi, Y., Rudic, R.D., Daumerie, G., Fulton, D., Sessa, W.C., and Walsh, K. (2000). Acute modulation of endothelial Akt/PKB activity alters nitric oxide–dependent vasomotor activity in vivo. J. Clin. Invest. 106, 493–499.

Mayet, J., and Hughes, A. (2003). Cardiac and vascular pathophysiology in hypertension. Heart Br. Card. Soc. 89, 1104–1109.

McVeigh, G.E., Allen, P.B., Morgan, D.R., Hanratty, C.G., and Silke, B. (2001). Nitric oxide modulation of blood vessel tone identified by arterial waveform analysis. Clin. Sci. Lond. Engl. 1979 100, 387–393.

Michaud, S.E., Dussault, S., Groleau, J., Haddad, P., and Rivard, A. (2006). Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species. J. Mol. Cell. Cardiol. 41, 275–284.

Mitchell BM , Dorrance AM, Webb RC. GTP cyclohydrolase 1 inhibition attenuates vasodilation and increases blood pressure in rats. (2003) Am J Physiol Heart Circ Physiol. 285(5):H2165-70.

Montecinos, V., Guzmán, P., Barra, V., Villagrán, M., Muñoz-Montesino, C., Sotomayor, K., Escobar, E., Godoy, A., Mardones, L., Sotomayor, P., et al. (2007). Vitamin C is an essential antioxidant that enhances survival of oxidatively stressed human vascular endothelial cells in the presence of a vast molar excess of glutathione. J. Biol. Chem. 282, 15506–15515.

Nozik-Grayck E, Woods C, Taylor JM *et al.* Selective depletion of vascular EC-SOD augments chronic hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol. (2014) 307(11):L868-76.

Ohara, Y., Peterson, T.E., and Harrison, D.G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. J. Clin. Invest. 91, 2546–2551.

Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui, H., Sakurai, H., Yoshida, Y., et al. (2002). Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. J. Clin. Invest. 110, 331–340.

Park, W.H. (2013). The effects of exogenous H2O2 on cell death, reactive oxygen species and glutathione levels in calf pulmonary artery and human umbilical vein endothelial cells. Int. J. Mol. Med. 31, 471–476.

Paulis, L., Zicha, J., Kunes, J., Hojna, S., Behuliak, M., Celec, P., Kojsova, S., Pechanova, O., and Simko, F. (2008). Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor. Hypertens. Res. Off. J. Jpn. Soc. Hypertens. 31, 793–803.

Rajagopalan, S., Kurz, S., Münzel, T., Tarpey, M., Freeman, B.A., Griendling, K.K., and Harrison, D.G. (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. J. Clin. Invest. 97, 1916–1923.

Santos-Parker, J.R., LaRocca, T.J., and Seals, D.R. (2014). Aerobic exercise and other healthy lifestyle factors that influence vascular aging. Adv. Physiol. Educ. 38, 296–307.

Schuppe, I., Moldéus, P., and Cotgreave, I.A. (1992). Protein-specific S-thiolation in human endothelial cells during oxidative stress. Biochem. Pharmacol. 44, 1757–1764.

Scotland RS, Madhani M, Chauhan S *et al.* Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. (2005) Circulation. 111(6):796-803.

Sélley, E., Kun, S., Szijártó, I.A., Laczy, B., Kovács, T., Fülöp, F., Wittmann, I., and Molnár, G.A. (2014). Exenatide induces aortic vasodilation increasing hydrogen sulphide, carbon monoxide and nitric oxide production. Cardiovasc. Diabetol. 13, 69.

Shinozaki, K., Nishio, Y., Okamura, T., Yoshida, Y., Maegawa, H., Kojima, H., Masada, M., Toda, N., Kikkawa, R., and Kashiwagi, A. (2000). Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ. Res. 87, 566–573.

Siddiqui, A. (2011). Effects of Vasodilation and Arterial Resistance on Cardiac Output. J. Clin. Exp. Cardiol. 02.

Sindler, A.L., Devan, A.E., Fleenor, B.S., and Seals, D.R. (2014). Inorganic nitrite supplementation for healthy arterial aging. J. Appl. Physiol. Bethesda Md 1985 116, 463–477.

Silva, B.R., Pernomian, L., and Bendhack, L.M. (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. Front. Physiol. 3, 441.

Song, P., Wu, Y., Xu, J., Xie, Z., Dong, Y., Zhang, M., and Zou, M.-H. (2007). Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. Circulation 116, 1585–1595.

Song, P., Xie, Z., Wu, Y., Xu, J., Dong, Y., and Zou, M.-H. (2008). Protein kinase Czeta-dependent LKB1 serine 428 phosphorylation increases LKB1 nucleus export and apoptosis in endothelial cells. J. Biol. Chem. 283, 12446–12455.

Stamler, J.S., Loh, E., Roddy, M.A., Currie, K.E., and Creager, M.A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 89, 2035–2040.

Su, Y., Liu, X.-M., Sun, Y.-M., Jin, H.-B., Fu, R., Wang, Y.-Y., Wu, Y., and Luan, Y. (2008). The relationship between endothelial dysfunction and oxidative stress in diabetes and prediabetes. Int. J. Clin. Pract. 62, 877–882.

Su, Y., Qadri, S.M., Wu, L., and Liu, L. (2013). Methylglyoxal modulates endothelial nitric oxide synthase-associated functions in EA.hy926 endothelial cells. Cardiovasc. Diabetol. 12, 134.

Talukder MA, Johnson WM, Varadharaj S *et al.* Chronic cigarette smoking causes hypertension, increased oxidative stress, impaired NO bioavailability, endothelial dysfunction, and cardiac remodeling in mice. (2011) Am J Physiol Heart Circ Physiol. 300(1):H388-96.

Tatham, A.L., Crabtree, M.J., Warrick, N., Cai, S., Alp, N.J., and Channon, K.M. (2009). GTP cyclohydrolase I expression, protein, and activity determine intracellular tetrahydrobiopterin levels, independent of GTP cyclohydrolase feedback regulatory protein expression. J. Biol. Chem. 284, 13660–13668.

Tiefenbacher CP, Chilian WM, Mitchell M *et al.* Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. (1996) Circulation. 94:1423–1429.

Uruno, A., Sugawara, A., Kanatsuka, H., Kagechika, H., Saito, A., Sato, K., Kudo, M., Takeuchi, K., and Ito, S. (2005). Upregulation of nitric oxide production in vascular endothelial cells by all-trans retinoic acid through the phosphoinositide 3-kinase/Akt pathway. Circulation 112, 727–736.

Vásquez-Vivar J, Martásek P, Whitsett J *et al.* The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. (2002) Biochem J. 362(Pt 3):733-9.

Wang, S., Xu, J., Song, P., Wu, Y., Zhang, J., Chul Choi, H., and Zou, M.-H. (2008). Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. Hypertension 52, 484–490.

Whitsett, J., Picklo, M.J., and Vasquez-Vivar, J. (2007). 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation. Arterioscler. Thromb. Vasc. Biol. 27, 2340–2347.

de Wijs-Meijler DP, Duncker DJ, Tibboel D, Schermuly RT, Weissmann N, Merkus D, Reiss IKM. Oxidative injury of the pulmonary circulation in the perinatal period: Short- and long-term consequences for the human cardiopulmonary system. Pulm Circ. 2017 1;7(1):55-66. doi: 10.1086/689748. eCollection 2017 Mar.

Wilkinson, I.B., MacCallum, H., Cockcroft, J.R., and Webb, D.J. (2002). Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br. J. Clin. Pharmacol. 53, 189–192.

Zhang, W., Han, Y., Meng, G., Bai, W., Xie, L., Lu, H., Shao, Y., Wei, L., Pan, S., Zhou, S., et al. (2014). Direct renin inhibition with aliskiren protects against myocardial ischemia/reperfusion injury by activating nitric oxide synthase signaling in spontaneously hypertensive rats. J. Am. Heart Assoc. 3, e000606.

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vásquez-Vivar, J., and Kalyanaraman, B. (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic. Biol. Med. 34, 1359–1368.

Zou, M.-H., Hou, X.-Y., Shi, C.-M., Nagata, D., Walsh, K., and Cohen, R.A. (2002). Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J. Biol. Chem. 277, 32552–32557.

Zweier JL, Chen CA, Druhan LJ. S-glutathionylation reshapes our understanding of endothelial nitric oxide synthase uncoupling and nitric oxide/reactive oxygen species-mediated signaling. (2011) Antioxid Redox Signal. 14(10):1769-75.