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Review

Redox regulation of vascular prostanoid synthesis by the nitric oxide-superoxide system ☆

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Abstract

Oxygen is involved in cell signaling through oxygenases and oxidases and this applies especially for the vascular system. Nitric oxide (·NO) and epoxyarachidonic acids are P450-dependent monooxygenase products and prostacyclin is formed via cyclooxygenase and a heme-thiolate isomerase. The corresponding vasorelaxant mechanisms are counteracted by superoxide which not only traps ·NO but through the resulting peroxynitrite blocks prostacyclin synthase by nitration of an active site tyrosine residue. In a model of septic shock, this leads to vessel constriction by activation of the thromboxane A_2–prostaglandin endoperoxide H_2 receptor. This sequence of events is part of endothelial dysfunction in which the activated vascular smooth muscle counteracts and regenerates vessel tone by cyclooxygenase-2-dependent prostacyclin synthesis. Peroxynitrite was found to activate cyclooxygenases by providing the peroxide tone at nanomolar concentrations. Such new insights into the control of vascular function have allowed us to postulate a concept of redox regulation in which a progressive increase of superoxide production by NADPH-oxidase, mitochondria, xanthine oxidase, and even uncoupled NO-synthase triggers a network of signals originating from an interaction of ·NO with superoxide.

Keywords: Prostacyclin synthase; Nitration; Nitric oxide; Prostaglandin E_2; Peroxynitrite; Cyclooxygenase; Endothelium; Smooth muscle

Oxygen is essential for higher organisms and has unique electronic properties to fulfill its large diversity of functions. The use of the ^{18}O-isotope 50 years ago has allowed us to differentiate between oxidase and oxygenase reactions. In oxidase reactions, electrons are transferred to oxygen whereas oxygenase reactions activate and insert oxygen in a molecule including reactions such as hydroxylation, epoxygenation, etc. However, many reactions involving oxygen are of transient state with unstable products or with a rapid exchange of oxygen, which further limits and complicates the analytical detection of such intermediates. A good example is the control of oxygen supply to the tissues.

It has turned out that the dynamics of blood flow are regulated by a complex network of messengers. The character of the vasculature as a system which has to respond to various environmental challenges requires feed-back or feed-forward control mechanisms and it is the purpose of this review to point out new aspects of interactions between prostanoids as a major group of signaling molecules and the nitric oxide (·NO) pathway as an equally potent but highly diverse regulatory mechanism for vascular tone. The complexity and apparent contradictions arise from several chemical reactions between ·NO and reactive oxygen species.

Superoxide anion ($\cdot O_2^-$) is emerging as an effective trapping agent for ·NO. The resulting peroxynitrite anion ($ONOO^-$) was recently found to have new regulatory properties via nitration of a protein-bound Tyr-residue in

☆ *Abbreviations:* ·NO, nitric oxide; $\cdot O_2^-$, superoxide; PGI_2, prostacyclin; PGIS, prostacyclin synthase; NOS, NO-synthase; COX, cyclooxygenase; SOD, superoxide dismutase; PN, peroxynitrite; EC, endothelial cell; SMC, smooth muscle cell; CYP, cytochrome P450; TxA_2, thromboxane A_2; PGH_2, prostaglandin endoperoxide H_2; LPS, lipopolysaccharide; TP, thromboxane A_2 receptor; PGE_2, prostaglandin E_2; ICAM, intercellular adhesion molecule; VCAM, vascular adhesion molecule; EET, epoxyeicosatrienoic acid; EDHF, endothelium-derived hyperpolarizing factor.

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prostacyclin synthase and Mn-superoxide dismutase, linking the prostanoid and the ·NO pathways. This has added substance to the new concept of redox signaling (for review, see [1]). Peroxynitrite is also involved in S-nitrosations as another new posttranslational modification and it participates in providing the peroxide tone for cyclooxygenases.

Regulation of vascular tone

Under physiological conditions contraction and relaxation of vascular smooth muscle cells (SMC) is controlled by a monolayer of endothelial cells which form a barrier between the media of the vessels and the circulating blood. By shear stress or agonist stimulation (e.g., acetylcholine, angiotensin II) the endothelium releases its autacoids ·NO and prostacyclin (PGI_2). These mediators activate guanylyl cyclase and adenylyl cyclase, respectively, resulting in a decrease of intracellular $[Ca^{2+}]_i$ levels in SMC causing blood vessel relaxation (for details refer to Fig. 1) [2].

At least one additional backup system for ·NO- and PGI_2-mediated relaxations exists in which the monooxygenation by cytochrome P450 2C9 of arachidonate to regio- and stereospecific epoxyeicosatrienoic acids (EETs) causes a hyperpolarization of smooth muscle [3]. Thereby, EETs enhance the open probability of a calcium-dependent K^+ channel (K^+_{Ca}) and activate the Na^+-K^+-ATPase leading to vasorelaxation. This so-called endothelium-derived

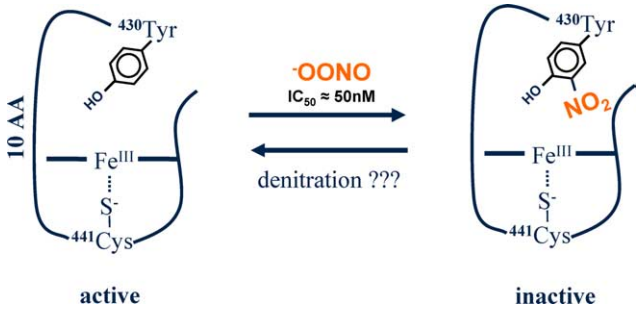

Fig. 2. Hypothetical scheme for prostacyclin synthase nitration. Inhibition originates from sterical hindrance of substrate access, since Tyr430Phe substitution is not affecting PGI_2 synthase activity (T. Tanabe, unpublished).

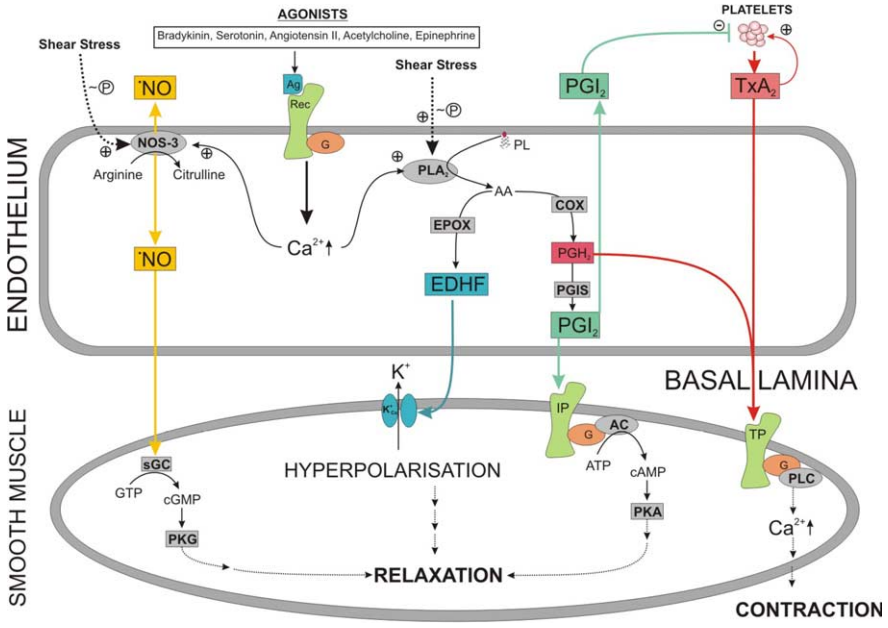

Fig. 1. Biochemical pathways in the regulation of vascular tone. Agonists such as angiotensin II or bradykinin bind to their transmembrane receptors and lead to an increase in $[Ca^{2+}]_i$. This, via Ca^{2+}/calmodulin interaction, leads to the activation of NOS III that generates the lipophilic and free diffusible ·NO from L-arginine in an oxygen-dependent manner. This gaseous molecule passes freely the endothelial membrane and activates the heme-regulatory subunit of soluble guanylyl cyclase in smooth muscle cells. Activation of the enzyme causes conversion of GTP to cGMP, a potent second messenger that triggers the activation of cGMP-dependent kinases (PKGs). Thus, a complex phosphorylation cascade is initiated and results in the lowering of $[Ca^{2+}]_i$ levels and relaxation of the actin cytoskeleton in SMC. Furthermore, the agonist-triggered $[Ca^{2+}]_i$ increase in endothelial cell liberates arachidonic acid from the membrane phospholipid pool mediated by acting in a phospholipase A_2. Free arachidonic acid serves as substrate for epoxygenase or cyclooxygenase. The cyclooxgenase-dependent pathway generates the intermediate PGH_2, which is further converted by PGI_2 synthase to PGI_2. Under defined conditions PGH_2 (see text for details) can accumulate, diffuse across the basal lamina, and stimulate the TP-receptor on SMC. This activates phospholipase C and leads via the PI-response to a raise in $[Ca^{2+}]_i$ and subsequent SMC constriction. PGI_2 acts synergistically to ·NO and thus causes by activation of its G-protein coupled receptor (seven membrane spanning) an activation of adenylyl cyclase. Rising levels of cAMP trigger protein kinase A and finally relax the vasculature. Also, the endothelium senses blood pressure and blood flow through mechanoreceptors coupled to the cytoskeleton, which activate the production of ·NO and PGI_2. Both, ·NO and PGI_2, prevent platelet aggregation, adhesion of leukocytes, apoptosis, and proliferation of smooth muscle cells. *Abbreviations:* AA, arachidonic acid; Ag, agonist; AC, adenylyl cyclase; EPOX, epoxygenase; G, G-protein, K^+_{Ca}, calcium-dependent K^+ channel; Rec, receptor; P, protein phosphorylation; PGIS, PGI_2 synthase; PKA, A-kinase; PKG, G-kinase; PL, phospholipids; PLA_2, phospholipase A_2; PLC, phospholipase C; sGC, soluble guanylyl cyclase.

hyperpolarizing factor (EDHF) is suppressed in the presence of the endothelium-derived autacoids ·NO and PGI_2. Likewise, ·NO is blocking the corresponding CYP2C9 epoxygenase, which represents a mechanism of down-regulation to the physiological state. Besides EETs hydrogen peroxide is discussed to be a second EDHF [4].

Since PGI_2 synthase, like the NO-synthases, is also a heme-thiolate enzyme, it is remarkable that all three relaxation mechanisms involve P450 enzymes and require oxygen as a cosubstrate. Under conditions of prolonged anoxia or hypoxia, oxygen becomes limited and synthesis of these mediators is severely impaired. In contrast to ·NO and PGI_2, EETs can be stored by incorporation into the membrane phospholipid pool. Thus, even under limiting oxygen supply, nature has developed a backup system to guarantee optimal perfusion by the phospholipase A_2-catalyzed release of EETs from phospholipids [5].

An even more powerful response to a lack of oxygen is the recently described reduction of nitrite to ·NO by the respiratory chain [6]. A shift of the redox equilibria within the chain to a reduced state facilitates the one-electron reduction of nitrite to ·NO whereas under high energy demand and sufficient oxygen supply NO may be reoxidized to nitrite by cytochrome *c* oxidase [7].

The major opponents of endothelium-derived autacoids are belong platelet activating factor (PAF), leukotriene C_4, endothelin, 15-hydroxy-9,11-endoperoxy arachidonic acid (PGH_2), or platelet-derived thromboxane (TxA_2). TxA_2 synthase is also a heme-thiolate enzyme but its product is a strong antagonist to PGI_2 so that both prostanoids form a Yin-yang system. The TxA_2 receptor (TP) is located on SMC but also on the endothelium where it causes contraction which may participate in the opening of the endothelial barrier. Emphasis has to be spent on the fact that the substrate for PGI_2 and TxA_2 synthase, PGH_2, also stimulates the TP-receptor. In the past, several drug companies developed potent TxA_2 synthase inhibitors, which were proven to be largely inefficient in restoring vascular homeostasis in contrast to TxA_2/PGH_2 receptor blockers. This previously not understood dual receptor specificity found a likely explanation by our finding of a nitration and inhibition of PGI_2 synthase by $ONOO^-$ [8]. After inhibition of endothelial PGI_2 synthase, the unmetabolized substrate PGH_2 accumulates and triggers the SMC TP-receptor, thus causing constriction of the vasculature.

Superoxide as the key to redox regulation—the role of oxidases

In the past, the physiological functions of $\cdot O_2^-$ as another low molecular weight radical were mostly underestimated. As a member of the ''reactive oxygen species'' (ROS) which include OH-radicals, H_2O_2, and alkoxy- and peroxy-radicals, it has been regarded as a nasty, life-threatening, and destructive oxygen-derived toxicant [9–11]. At a first sight such properties seem to be supported by its role in leukocytes and macrophages, but without considering that not $\cdot O_2^-$ but hydrogen peroxide, hypochlorite or peroxynitrite are the ultimate oxidants in such defense processes. Even the long known thermodynamics of superoxide and its use as a reductant for cytochrome *c* could not change its image as a radical of oxidative power [12,13]. One reaction, however, has been supportive for a role as a dangerous molecule in the cell: its reduction of protein-bound Fe^{3+}. Following reduction by $\cdot O_2^-$ ferritin or iron–sulfur clusters (e.g., aconitase and fumarase) release free ferrous iron into the cytoplasm which then can initiate the Haber–Weiss cycle to generate the highly reactive OH-radical [14,15]. This reaction leads to cellular toxicity and thus explains the existence of superoxide dismutases (Cu,Zn-SOD, Mn-SOD) as cellular antioxidants. SODs are essential for providing a low basal $\cdot O_2^-$ level in order to allow an additional and targeted formation of $\cdot O_2^-$ to act as a messenger system. On the one hand, $\cdot O_2^-$ was shown to inhibit the serine/threonine phosphatase calcineurin by targeting the binuclear Fe^{2+}-Zn^{2+} center at the active site [16] and on the other hand it was demonstrated to activate PKC via oxidation of its zinc finger [17]. These two enzymes are the link between $\cdot O_2^-$ as a messenger molecule and cellular phosphorylation cascades.

Enhanced $\cdot O_2^-$ formation in the cardiovascular system is mainly associated with cardiovascular complications such as atherosclerosis, ischemia–reperfusion injury, diabetes, hyperlipidemia, hypertension, etc. Nevertheless, latest results indicated that the relative concentration of the radical is of importance for its role as a signaling molecule. At low timely and locally controlled concentrations $\cdot O_2^-$ acts as a messenger molecule whereas at higher concentrations it impairs redox regulation and guides the vasculature into a dysfunctional state. This could either result from lowered cellular antioxidant defense mechanisms or from an activation of cellular $\cdot O_2^-$ sources.

NADPH-oxidases (NOX) have evolved as a large family containing so far seven members, NOX1-5 (homologs of $gp91^{phox}$) [18,19], and the two long homologs of NADPH-oxidases [20].

CYP2C9 as the putative EDHF synthase also produces $\cdot O_2^-$ as an unwanted by-product, but possible implications on vascular function still are speculative [21].

Also NO-synthases can synthesize $\cdot O_2^-$ which represents an enzymatic malfunction. Oxidation of the cofactor H_4B to H_2B, limitation of L-arginine supply, monomerization and zinc release, or alterations in the heat shock protein 90 can cause uncoupling and therefore $\cdot O_2^-$ release by NO-synthases. An uncoupled eNOS is often observed in endothelial dysfunction and could be a consequence of prolonged oxidative stress [22–24].

A very potent source of $\cdot O_2^-$ is xanthine oxidase, which derives from oxidative and/or proteolytic conversion of xanthine dehydrogenase. Oxidatively caused lesions after ischemia–reperfusion have mainly been attributed to this enzyme and also a contribution in severe endotoxemia was observed [25,26].

Finally, mitochondria can generate $^{\cdot}O_2^-$, originating from autoxidation of electron transport chain components or electron leaks at complex I or III. This process can be triggered by cellular signaling cascades, e.g., ceramide release, and hence rather seems to be an enhancer in redox regulation than a malregulation of the respiratory chain.

Since $^{\cdot}O_2^-$ appears too weak as a direct oxidant, a derived peroxide would be a better candidate for mediating oxidative effects on regulatory biomolecules. Hydrogen peroxide is also rather inert in the absence of metals whereas $ONOO^-$, originating from the reaction of $^{\cdot}NO$ and $^{\cdot}O_2^-$, represents an unstable and reactive molecule [10]. Under physiological conditions, $ONOO^-$ levels are kept in the nanomolar range by cellular antioxidant systems to avoid damages of cellular macromolecules. However, with increasing levels of $^{\cdot}O_2^-$ and $^{\cdot}NO$ and the involvement of metal catalysis, specific oxidations at proteins can occur (sulfenic acid formation, sulfoxidation, and zinc finger oxidation) which lead to posttranslational protein modifications and activity changes.

Prostacyclin synthase nitration and consequences for vascular homeostasis

In presence of low levels of $ONOO^-$ ($IC_{50} \approx 50$ nM) a Tyr-residue at the active site of PGI_2 synthase becomes nitrated and inhibits the active site [27]. Recently, the crystal structure of PGIS was resolved by Raman et al. (personal communication). As expected, Tyr-430 faces the active site so that nitration of this residue would sterically hamper the access of PGH_2 to the heme catalytic site (Fig. 2).

By using other P450 proteins as models [28], it became clear that the heme catalyzes the nitration leading to high rates of nitration which exceed the inactivation of $ONOO^-$ by the antioxidant potential of the cell. Inhibition of PGI_2 synthase causes PGH_2 to accumulate which, as mentioned before, could activate the TP-receptor exerting a dual specificity for TxA_2 and PGH_2 [29,30]. This can be demonstrated by the contraction of the smooth muscle which is completely prevented by a TP-receptor antagonist [31]. Thus, a pathophysiological situation would arise. PGI_2 synthase was found nitrated in atherosclerotic blood vessels [32], in diabetes [33,34], after anoxia–reperfusion of coronary artery segments [35], after nitroglycerol treatment causing cross-tolerance in rats [36], and in a model of septic shock [37]. Evidence comes also from SOD $1^{-/-}$ knock out animals which exert an impaired COX- and NOS-dependent vasorelaxation [38]. Such conditions are those of ''endothelial dysfunction'' and we therefore have proposed PGI_2 synthase nitration as a hallmark of a dysfunctional endothelium. Accordingly, formation of $^{\cdot}O_2^-$ radicals could cause the transition from a physiological to a pathophysiological state which is summarized in a simplified scheme (Fig. 3).

It should be noted that the transition to a dysfunctional state of the endothelium would require fluxes of $^{\cdot}NO$ and $^{\cdot}O_2^-$ in the range of 30–100 nM to yield $ONOO^-$. This may be limited to cellular compartments like caveolae where NOS-3 and PGI_2 synthase could be found. If lower levels of $^{\cdot}O_2^-$ are produced, one can even observe an activation of PGI_2 synthesis which will be discussed later. Consequently, endothelial dysfunction can be preceded by an activated state. This is subject of ongoing studies and will be further explained in connection with a model of septic shock.

PGI_2 synthase nitration in a model of septic shock—endothelial dysfunction versus endothelial cell activation

Endotoxin (LPS) represents a powerful model for bacterial infections, although it has to be reminded that the presence of serum essentially alters its biological activity. Bovine coronary segments when exposed to LPS (10 μg/ml) for 40–60 min change dramatically their behavior against a stimulus of angiotensin II. Whereas the first contraction phase proceeds normally the subsequent relaxation to the baseline was converted to a continuous contraction resembling a vasospasm [37]. This second contraction could be blocked completely by a TP-blocker and since no TxA_2 was formed it must have been mediated by PGH_2. Under these conditions PGI_2 synthase was nitrated and inhibited, and this could be blocked by an NO-synthase inhibitor and by polyethylenglycolated (PEG)-SOD (see Fig. 1). In addition, both the nitration and the vasospasm were sensitive to allopurinol, suggesting that the $^{\cdot}O_2^-$ source was xanthine oxidase [37]. Considering that LPS required at least 40 min to produce its effect, the crucial release of $^{\cdot}O_2^-$ by xanthine oxidase might have been triggered either by the conversion of xanthine dehydrogenase to its oxidase form or through the formation of its substrates hypoxanthine/xanthine or by both. Since mitochondria are sensitive to an oxidative cellular environment [39,40], ATP breakdown may also represent an essential step for $^{\cdot}O_2^-$ formation by xanthine oxidase.

This model of LPS-exposed bovine coronary arteries certainly has its limitations when the time period from 2 to 12 h LPS exposure is of interest. This is the time in which early genes like ICAM, VCAM or NOS-2 and COX-2 are induced and when these enzymes take over the progression of inflammation.

Smooth muscle cell PGI_2 synthase—a resting pool of PGI_2

The finding of a functionally impaired endothelial PGI_2 synthase in LPS-exposed vessels was at first sight in contrast to clinical observations demonstrating highly elevated levels of 6-keto-$PGF_{1\alpha}$ in the plasma of septic shock patients [41]. To explain this contradiction, we hypothesized that SMC could have been the source of PGI_2 after induction of cyclooxygenase-2 (COX-2) which then could provide PGH_2 for the constitutively expressed PGI_2 synthase in SMC [42,43]. This could be fully confirmed by using bovine and human SMC in culture [42]. Such high levels of PGI_2 would counteract effectively the vasospasm caused by PGH_2 and prevent platelet aggregation as well as prolif-

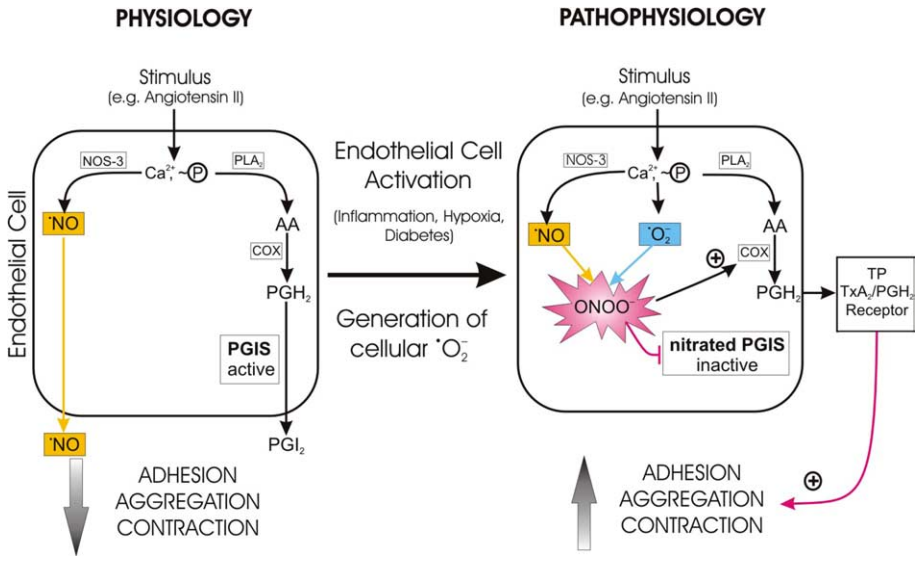

Fig. 3. Effects of superoxide generation on vascular tone. Generation of $^{\cdot}O_2^-$ will lower the bioavailability of free ·NO, leading to enhanced $ONOO^-$ formation. $ONOO^-$ nitrates and inactivates PGI_2 synthase at a concentration of about 50 nM. As a consequence of PGI_2 synthase inhibition and activation of cyclooxygenase by $ONOO^-$ (refer to "peroxide tone") PGH_2 will accumulate. Thus, two mediators of vascular homeostasis are diminished and increased levels of PGH_2 cause vasoconstriction and favor adhesion and thrombus formation. *Abbreviations:* P, protein phosphorylation; PLA_2, phospholipase A_2; TP, thromboxane A_2/PGH_2 receptor.

eration of SMC. The dysfunction of the endothelium in the first two hours after LPS is therefore followed by a second phase in which SMC replace the dysfunctional endothelium with regard to PGI_2 formation.

Of note, the PGI_2 release was observed by bovine and human SMC, but not by rat SMC which produced ·NO instead [44]. This indicates a species-dependency and has to be considered in the therapy of shock related vascular disorders.

Peroxynitrite as provider of the peroxide tone

The release of high amounts of PGI_2 in a COX-2-dependent manner in SMC allowed an investigation on the regulation of PGI_2 release in these cells. To our surprise the addition of $ONOO^-$ did not inhibit PGI_2 synthase by nitration but rather doubled its activity [45]. Since COX-1 as well as COX-2 require peroxides for activation, we tested whether this so called "peroxide tone" was not saturated. Indeed, H_2O_2 caused also a doubling of activity indicating non-saturating conditions for the intracellular peroxide level. Already 2 nM of arachidonic acid peroxides [46] had been reported sufficient, suggesting that the peroxide tone in SMC was kept around 1 nM in order to explain the doubling of PGI_2 output. An obviously strong peroxidase activity in SMC would also explain the lack of nitration by added $ONOO^-$ and this was confirmed by employing redox cyclers to weaken the reductive power of these cells (unpublished P. Zimmerling et al.).

The ability of added $ONOO^-$ to enhance the peroxide tone provoked the idea that $ONOO^-$ could also provide the endogenous peroxide tone for COX-2 in SMC. Inhibition of NOS activity indeed lowered the yield of PGI_2 [45]. As a physiological source for $^{\cdot}O_2^-$ NADPH-oxidase seemed a suitable candidate and apocynin as inhibitor also lowered the activity. Using uric acid as a selective scavenger for $ONOO^-$ we observed an effective inhibition which strengthened the hypothesis that nanomolar levels of $ONOO^-$ are required to set up the peroxide tone as summarized in Fig. 4.

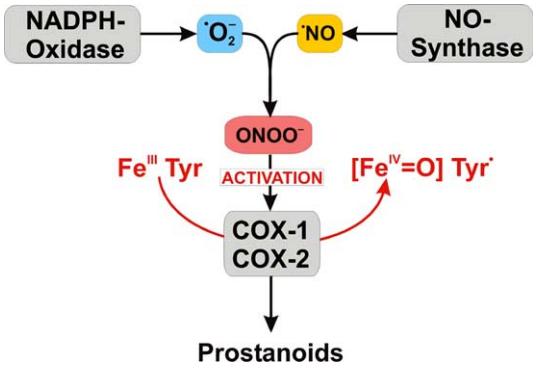

Fig. 4. Peroxynitrite as provider of the peroxide tone. The cyclooxygenases contain two distinct catalytically active subunits, the peroxidase and the cyclooxygnase domain. The cyclooxygenase domain catalyzes the insertion of two oxygen molecules at the 9, 11, and 15 position of arachidonic acid to form the prostaglandin endoperoxide G_2. In the next step of catalysis, the peroxide at position 15 is reduced to an alcohol by a peroxidase activity. However, to initiate catalysis (resting state; Fe^{III} Tyr) by the cyclooxygenase activity a tyrosyl radical (Tyr385) is required (oxyferryl plus tyrosyl radical; $[Fe^{IV}=O]$ Tyr·). The required concentrations of peroxides differ among the two isoforms (COX-1 $\approx$ 21 nM; COX-2 $\approx$ 2 nM). Initiation and maintenance of catalysis by peroxides is summarized with the term "peroxide tone." We have demonstrated that in smooth muscle cells $ONOO^-$ is an endogenous and efficient provider of the peroxide tone.

A limitation of PGI_2 synthesis by a not fully saturated peroxide tone could also explain earlier data on a stimulation of 6-keto-$PGF_{1\alpha}$ formation after 5 min of LPS exposure in the bovine coronary segment model [37]. Obviously, the onset of $^{\cdot}O_2^-$ release elevates $ONOO^-$ levels slowly and first saturates the peroxide tone under stimulation of PGH_2 release and only at higher fluxes of $^{\cdot}O_2^-$ and $^{\cdot}NO$ leads to inhibition by nitration.

As can be expected many unanswered questions remain. $ONOO^-$ has to be formed from $^{\cdot}NO$ and $^{\cdot}O_2^-$ at equal rates and it is still obscure how this is adjusted in the cell. We favor the idea that NOS-activity (probably NOS-1) can be upregulated in parallel to $^{\cdot}O_2^-$-generation but this remains to be verified.

Acknowledgments

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