Regulation of ion channel structure and function by reactive oxygen-nitrogen species

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Matalon, Sadis, Karin M. Hardiman, Lucky Jain, Douglas C. Eaton, Michael Kotlikoff, Jerry P. Eu, Junhui Sun, Gerhard Meissner, and Jonathan S. Stamler. Regulation of ion channel structure and function by reactive oxygen-nitrogen species. Am J Physiol Lung Cell Mol Physiol 285: L1184–L1189, 2003; 10.1152/ajplung.00281.2003.—Ion channels subserve diverse cellular functions. Reactive oxygen and nitrogen species modulate ion channel function by a number of mechanisms including 1) transcriptional regulation of gene expression, 2) posttranslational modifications of channel proteins, i.e. nitrosylation, nitration, and oxidation of key amino acid residues, 3) by altering the gain in other signaling pathways that may in turn lead to changes in channel activity or channel gene expression, and 4) by modulating trafficking or turnover of channel proteins, as typified by oxygen radical activation of NF-kB, with subsequent changes in proteasomal degradation of channel degradation. Regardless of the mechanism, as was discussed in a symposium at the 2003 Experimental Biology Meeting in San Diego, CA, changes in the cellular level of reactive oxygen and nitrogen species can have profound effects on the activity of ion channels and cellular function.

ION CHANNELS are one of the major mechanisms for transducing external signals across the cell membrane to the cell interior. Therefore, intracellular signaling mechanisms that control their activity are extremely important to normal cellular function and cellular homeostasis. Because of the importance of ion channels, many traditional cellular effectors modulate their function; however, the role of reactive oxygen and nitrogen species (ROS and RNS) in altering ion channel activity has only recently been recognized. Ion channel modulation by such reactive species may occur in several different ways. The simplest is through direct post-translational modification of channel proteins. Nitrosylation, nitration, and oxidation of key amino acid residues are examples of such modification. Alternatively, reactive species may alter the activity of other signaling mechanisms that secondarily lead to changes in channel activity or channel gene expression. An example of this is the activation of the MAPK pathways by ROS or the G kinase pathway by nitric oxide (NO). Finally, there are other, more complex mechanisms mediated through alterations in trafficking or turnover of channel proteins, as typified by oxygen radical activation of NF-kB, with subsequent changes in proteasomal degradation of channel degradation. Regardless of the mechanism, as discussed in this symposium, changes in the cellular level of ROS and RNS can have profound effects on the activity of ion channels and cellular function. The four speakers (Drs. Eaton, Kotlikoff, Stamler, and Matalon) discussed their most recent findings concerning the effects of reactive oxygen-nitrogen intermediates on Ca2+, K+, Na+, and Cl− channels.

ROS/RNS DECREASE CFTR EXPRESSION AND CAMP-MEDIATED CHLORIDE SECRETION IN AIRWAY EPITHELIUM

Because of their location, airway and alveolar epithelial cells are often exposed to increased intracellular and extracellular concentrations of ROS and RNS present in cigarette smoke, environmental pollutants, and oxidant gases, or generated by activated inflammatory cells. ROS are formed as intermediates of the incomplete reduction of oxygen in mitochondrial electron-transport systems, by microsomal metabolism of endogenous compounds and xenobiotics, or by various enzymatic generators, such as xanthine oxidase. Neutrophils and other inflammatory cells generate and release ROS via an NADPH oxidase-dependent mech-

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anism, which is mediated by membrane receptor activation of protein kinase C and phospholipase C.

NO synthesis involves the five-electron oxidation of the guanidino nitrogen of L-arginine by NO synthases (NOS). The three enzymes that make NO (and related reactive species S-nitrosothiols and peroxynitrite) are the endothelial (eNOS), neuronal (nNOS), and inducible nitric oxide synthases (iNOS). Potential sources of RNS include rat-, mouse-, and human-activated alveolar macrophages, neutrophils, alveolar type II cells, and airway cells (13, 16, 17, 26, 34). Increased iNOS levels have been found in alveolar macrophages and human lung tissue obtained from patients with acute respiratory distress syndrome (14, 37) and other inflammatory lung diseases.

CFTR is a 1,480-amino acid protein and a member of the traffic ATPase family. It functions as a cAMP-regulated Cl⁻ channel and controls other ion conductive pathways, including epithelial Cl⁻ and Na⁺ channels as well as ATP transport. Cystic fibrosis is caused by defective CFTR function and is characterized by abnormal Na⁺ and Cl⁻ ion transport in several tissues, including the lungs, pancreas, gastrointestinal tract, liver, sweat glands, and male reproductive system (25).

Existing evidence indicates that acute exposure of airway and alveolar epithelial cells to NO, generated either endogenously or by chemical donors, stimulates Cl⁻ transport via cGMP-dependent mechanisms (6).

Similar effects were noted across intestinal cells as well as human lymphocytes. We became interested in the possible effects of prolonged exposure of ROS/RNS on Cl⁻ channels. We exposed polarized kidney epithelial cells (LLC-PK₁), which had been stably transduced with a cDNA encoding human wild-type CFTR under the control of a Zn²⁺-induced metallothionein promoter to 125 μM diethylenetriamine NONOate (DETA-NONOate; [NO] < 100 nM) at 37°C for 24 h. This treatment reduced both Zn²⁺- and -uninduced CFTR protein levels by 59 ± 5 and 67 ± 4% from their corresponding control values, respectively, by a cGMP-independent mechanism (22).

In our next series of experiments and its reactive intermediates, we investigated putative mechanisms by which NO modulate CFTR expression and function in CFTR-expressing airway epithelial cells. Immuno-precipitation followed by Western blot analysis, as well as immunocytochemical and cell surface biotinylation measurements, showed that incubation of both stably transduced (HeLa-3) and endogenous CFTR-expressing [16HEBE14o–, Calu-3, and mouse tracheal epithelial (MTE)] cells with 100 μM DETA-NONOate for 24–96 h decreased both intracellular and apical CFTR levels. Calu-3 and MTE cells, incubated with DETA-NONOate, but not with 100 μM 8-bromoguanosine 3',5'-cylic monophosphate (8-Br-cGMP) for 96 h, exhibited reduced cAMP-activated short-circuit currents (Isc) when mounted in Ussing chambers. Exposure of Calu-3 cells to NO donors resulted in the nitration of a number of proteins, including CFTR. Nitration was augmented by proteasome inhibition, suggesting a role for the proteasome in the degradation of nitrated proteins. Our studies demonstrate that levels of NO that are likely to be encountered in the vicinity of airway cells during inflammation may nitrate CFTR, resulting in enhanced degradation and decreased function (2). Protein nitration and oxidation by ROS/RNS in vitro have been associated with diminished function of a variety of crucial proteins present in the alveolar space, including surfactant protein A (50).

It is important to stress that our data do not imply that nitration is the only mechanism responsible for the decrease in CFTR level and function. In several systems, the biological effects of NO on transport proteins have been associated with the formation of nitrosothiols (RSNO). Once formed, RSNO adducts stabilize NO and may decrease its cytotoxic potential while maintaining or promoting its bioactivity. For example, it has been suggested that S-nitrosoglutathione (GSNO), which is present endogenously in the airways, can increase expression of the mature and functional CFTR (49). This also appears to be the case in neurons where certain RSNO, generating nitrosothiols but not NO per se, resulted in S-nitrosylation of critical thiols at the N-methyl-d-aspartate (NMDA) receptor's redox modulatory site. S-nitrosylation of the NMDA receptor thereby prevents excess Ca²⁺ entry into cells and reduces the neurotoxicity associated with NO (29).

In summary, decreased levels and function of normal CFTR may account for some of the cystic fibrosis-like symptoms that occur in chronic inflammatory lung diseases associated with increased NO production.

MODULATION OF MAXI-K⁺ CHANNELS AND RYANODINE RECEPTORS BY NO

Calcium-activated K⁺ (maxi-K⁺) channels are a key signaling target in smooth muscle. This activity leads to a hyperpolarization that underlies vasodilation, bronchodilation, and effects on gastrointestinal, uterine, and urogenital tract smooth muscle. The direct effect of molecules such as NO on maxi-K⁺ channels has been studied in off-cell preparations, but such experiments often provide little information about the cellular context and physiological relevance of these interactions. Conversely, experiments in native cells are often confounded by effects on multiple ion channels and complex regulatory systems. To assess the mechanism of guanylyl cyclase and NO coupling to activation of maxi-K⁺ channels, the principal coupling molecules were reassembled in the Xenopus oocyte system. The α- and β-subunits of the human maxi-K⁺ channels were heterologously expressed in the Xenopus oocyte system. The α- and β-subunits of the human maxi-K⁺ channels were heterologously expressed, along with a receptor guanylyl cyclase, the rat atrial natriuretic peptide (ANP) receptor (31). Application of ANP allowed us to examine the mechanism of stimulatory coupling and to determine whether exposure to NO results in channel stimulation in the absence of this coupling system.

Stimulation of Xenopus oocytes with ANP resulted in a modest, but consistent, augmentation of maxi-K⁺ current. The maximum current was augmented by ~30%, with no effects on activation or inactivation
kinetics. This stimulatory effect was completely abolished by direct injection of an inhibitory peptide of protein kinase G (PKG), whereas the protein kinase A inhibitory peptide was without effect, indicating that stimulation of guanylyl cyclase resulted in PKG-dependent stimulation of the maxi-K⁺ current, without detectable cross talk. We next performed site-directed mutation of consensus phosphorylation sites on the α-subunit. Mutation of Ser855 and Ser869 to alanine mutation of consensus phosphorylation sites on the tectable cross talk. We next performed site-directed and Ca²⁺-subunit, whereas direct activation by NO is not ob-

stimulatory coupling occurs through activation of (end-

dependent mechanisms). Jain et al. (19) isolated rat ATII cells, patched them in the cell-attached mode, and recorded single channel activity before and after perfusion of these cells with GSNO and S-nitroso-N-acetylpencillamine. They reported that these agents increased ATII cell cGMP content and significantly reduced the open probability (Pₒ) of a 20-pS nonselective cation channel in cell-attached patches of ATII cells, that pretreatment with a PKG inhibitor prevented the inhibitory effects of GSNO on this channel, and that incubation of ATII cells with a cell-permeable analog of cGMP (8-Br-cGMP) also decreased the Pₒ. They concluded that NO decreased the activity of this channel by activating a cGMP-dependent protein kinase. Lazrak et al. (28) patched A549 cells using both the whole cell and cell-attached techniques and showed that exposure of these cells to 1-propanamine-3-(2-hydroxy-2-nitroso-1-propylhydrazine) (PAPANONOate) and spermine NONOate decreased selectively amiloride-sensitive whole cell currents via cGMP-dependent mechanisms.

In subsequent studies, Guo et al. (12) isolated rat ATII cells, cultured them on monolayers to confluence, mounted them in Ussing chambers, and measured Iₛₑ before and after addition of NO donors in the apical and basolateral compartments. NO, generated by spermine NONOate and PAPANONOate, decreased Iₛₑ across rat ATII cells with a half inhibitory concentra-
tion of 0.4 μM without affecting transepithelial resistance. NO also inhibited ~60% of the amiloride-sensi-
tive Iₛₑ across ATII cell monolayers after permeabiliza-
tion of the basolateral membrane with amphotericin B. However, in contrast to the findings of Jain et al. (19), incubation of ATII monolayers with 8-Br-cGMP (400 μM) did not decrease Iₛₑ. These results suggest that the effects of NO on amiloride-sensitive currents occurred through cGMP-independent mechanisms and were caused by direct interaction of reactive oxygen-nitro-
gen intermediates with channel or cytoskeletal pro-
tein(s). Differences among these studies may be the result of changes in the phenotype of ion channels as a result of culture.

ROS/RNS may interfere with Na⁺ transport across epithelial cells by damaging important structural proteins necessary for the proper function of these transporters. Compeau et al. (5) assessed changes in Na⁺ transport across monolayers of rat distal fetal epithelial cells after incubation of these cells with macrophages stimulated with endotoxin for 16 h. They reported a 75% decline in transepithelial resistance (Rₑ) and a selective 60% reduction in amiloride-sensitive Iₑsc. Single channel patch-clamp analysis demonstrated a 60% decrease in the density of a 25-pS nonselective cation channel present in the apical membrane of these cells. However, single channel conductance and Pₑ were not affected. A concurrent reduction in epithelial F-actin content suggested a role for actin depolymerization in mediating this effect. Incubation of cocultures with NGO-monomethyl-L-arginine, a NOS inhibitor, prevented the reduction in epithelial Iₑsc. These data indicate that ROS/RNS may affect amiloride-sensitive cation channels by reducing F-actin or other cytoskeletal structures.

In summary, existing evidence indicates that NO and related redox-active molecules modulate Na⁺ transport and the biophysical properties of cation channels of alveolar epithelial cells via cGMP-dependent and cGMP-independent mechanisms.

REDOX-BASED REGULATION OF SKELETAL MUSCLE RYR BY RNS

The traditional view that ROS and RNS are cytotoxic agents has now been superceded by the notion that these reactive molecules can transduce or regulate many physiological processes with remarkable specificities. For example, recent studies have demonstrated that transcriptional factors [e.g., OxyR (10, 24)], ion channels [e.g., olfactory cyclic nucleotide-gated channel (4)], and enzymes can be activated or regulated by RNS or ROS via redox-based modifications of specific thiols within these proteins. In this brief review, we summarize the results of a series of structure-function analyses on the skeletal muscle intracellular Ca²⁺ release channel, also known as type 1 ryanodine receptor (RyR1). These studies have not only revealed the precise nature of the reactions of RNS with specific classes of regulatory RyR1 thiols but they have also provided general mechanistic insight into how redox signaling messengers may influence Ca²⁺-dependent signaling.

Striated muscle contraction is initiated by the rapid release of Ca²⁺ from the sarcoplasmic reticulum (SR) via the RyR in response to a muscle action potential and is subsequently terminated by the resequestration of Ca²⁺ into SR by SR Ca²⁺-ATPase (9). Active muscle produces ROS and RNS that modulate its contraction and relaxation (9, 27, 35, 36). Skeletal muscle and cardiac muscle isoforms of RyR have been proposed as targets of endogenously produced RNS (that include NO, small-molecule S-nitrosothiols, and peroxynitrite) (1, 27) because these channels contain regulatory thiols that are susceptible to redox-based modifications by these molecules (38). Moreover, the colocalization of NO synthases and RyR within striated myocytes (38, 47) may allow RNS to react with regulatory thiols of RyR without affecting the overall redox state of the cell.

The skeletal muscle isoform of RyR contains 101 cysteine residues per homotetrameric subunit (45). With the use of a fluorescent-based assay, we found that the number of free thiols is dependent on O₂ tension (P₀₂), ratio of oxidized/reduced glutathione, and ambient concentration of ROS or RNS (42, 44, 46). RyR1 in SR vesicles has ~29 and 35 free thiols per subunit under ambient O₂ tension (P₀₂ ~ 150 mmHg) and tissue O₂ tension (P₀₂ ~ 10 mmHg), respectively. In the SR, but not in purified RyR1, the redox state of these six P₀₂-sensitive thiols is reversible. The presence of exogenously added glutathione (5 mM) or dithiothreitol (5 mM) does not affect the number of O₂-sensitive RyR1 thiols. The oxidation of RyR1 thiols at P₀₂ ~ 150 mmHg is associated with a significant increase in RyR1 channel activity (7). The physiological relevance of this oxidation-mediated change in activity is currently being elucidated (11, 18). As described below, however, this oxidized form of RyR1 is unresponsive to physiological concentrations of NO. Thus the results of ex vivo muscle contractility studies aimed to elucidate the effects of RNS in skeletal muscles need to be interpreted with caution, since many of these studies were carried out under supraphysiologically O₂ concentrations (usually P₀₂ ~ 700 mmHg) (27).

We found that NO had essentially no effect on RyR1 channel activity at P₀₂ ~ 150 mmHg unless NO concentrations exceeded 10 μM. In contrast, at P₀₂ ~ 10 mmHg (with RyR1 in the reduced state), physiological concentrations of NO (~1 μM) readily activated the RyR1, as indicated by the results of single channel measurements and [³H]ryanodine binding (7). With the use of a chemiluminescence method, we found that S-nitrosylation of one RyR1 thiol was responsible for the increase in channel activity at the lower O₂ tension.

Our subsequent work has focused on identifying the RyR1 thiol that is S-nitrosylated and the mechanism underlying the associated increase in activity. We observed that S-nitrosylation by NO required calmodulin and exerted effect by reversing the action of calmodulin on the channel (7). Because a single cysteine (C3635) is found within the calmodulin binding site of RyR1 (15, 33), we heterologously expressed full-length RyR1 in which C3635 was replaced by alanine. Unlike its wild-type counterpart, the C3635A mutant was neither activated nor S-nitrosylated by physiological concentrations of NO at P₀₂ ~ 10 mmHg, thus providing strong indication that C3635 is the NO-sensitive target in RyR1 (41). Furthermore, the slow-releasing NO donor N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethamine S-nitrosylated and activated wild-type RyR1, but not the C3635A mutant, independently of oxygen.
tension. Thus C3635 does not appear to be among the six to eight RyR1 thiols that are PO2 sensitive (44).

Under physiological or pathological conditions, NOS activity in skeletal muscle generates NO, small-molecule S-nitrosothiols (for example, GSNO), and peroxynitrite (a reaction product of NO and O2- (39)). 3-Morpholinosydnonimine (SIN-1), which generates mM irreversibly inhibited the channel, possibly due to GSNO (NO released for six to eight RyR1 thiols that are PO2 sensitive (44).

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dynamically alter the redox state of six to eight RyR1 thiols by NO/O2 oxidized) (42). The changes in RyR1 channel function by NO/O2 oxidized a single thiol within the calmodulin binding domain, thus reversing the inhibitory effect of calmodulin on the channel. These in vitro observations predict that the contractility of skeletal muscle groups enriched in NO synthesize (for example, the fast-twitch extensor digitorum longus muscles of rodents) should generate relatively more force under physiological tissue O2 tension (PO2 ≈ 10 mmHg). In addition, the identities of O2 tension-sensitive RyR1 thiols as well as the enzymatic system(s) in the SR that dynamically alter the redox state of six to eight RyR1 thiols in response to a change in O2 tension remain to be determined. GSNO and oxidized glutathione modify additional classes of thiols that may have a pathophysiological correlate in nitrosative and oxidative stress.

In summary, the results of our structure-function analyses demonstrate that RyR1 contains several different functional classes of thiols. Under physiologically relevant conditions, RyR is in a reduced form, and NO can S-nitrosylate a single thiol within the calmodulin binding domain, thus reversing the inhibitory effect of calmodulin on the channel. These in vitro observations predict that the contractility of skeletal muscle groups enriched in NO synthesize (for example, the fast-twitch extensor digitorum longus muscles of rodents) should generate relatively more force under physiological tissue O2 tension (PO2 ≈ 10 mmHg). In addition, the identities of O2 tension-sensitive RyR1 thiols as well as the enzymatic system(s) in the SR that dynamically alter the redox state of six to eight RyR1 thiols in response to a change in O2 tension remain to be determined. GSNO and oxidized glutathione modify additional classes of thiols that may have a pathophysiological correlate in nitrosative and oxidative stress.

REFERENCES


