Symposium
Thiol proteins: Structure and antioxidant and redox sensor activities

Coordenador: Ohara Augusto

Abstract of the talks:

**Oxidation of albumin thiol to sulfenic acid and to higher oxidation states**

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With the aim of understanding the interactions between reactive oxygen species and intravascular targets as well as identifying potential biomarkers of oxidative damage, we have focused on albumin, the most abundant protein in plasma. Previous work from our group showed that the single thiol in albumin (HSA-SH) was the preferential plasma target of reactive species. Indeed, HSA-SH reacted with hydrogen peroxide and peroxynitrite with rate constants of 2.26 and 3.8 x 10^3 M^-1 s^-1, respectively (pH 7.4, 37 ºC). The oxidation of HSA-SH did not lead to the formation of intermolecular albumin disulfides. Rather, sulfenic acid (HSA-SOH) could be detected. The sulfenic acid in HSA was remarkably stable, with ~15% decaying after 2h at 37ºC, and could be formed through both one- or two-electron pathways. In order to study its properties, we developed a technique based on the reaction with the chromophoric thiol thionitrobenzoate. This allowed us to quantify the formation of 0.14 ± 0.02 moles of HSA-SOH per mole of HSA, representing 29% of oxidized thiols, after reaction of 0.5 mM HSA with 4 mM hydrogen peroxide (4 min). In addition, we have adapted an hydrophobic interaction-anion exchange HPLC method for the analysis of the different oxidation states of albumin. In plasma samples and commercial albumin preparations, we were able to detect a reduced fraction (HSA-SH), a mixed disulfide fraction (HSA-SSR), an HSA-SOH fraction that reacted with thiols and a fourth fraction non-reducible by thiol reagents, that probably included higher oxidation states. Indeed, through mass spectrometry we evidenced the formation of sulfinic acid (HSA-SO$_2$H). We propose that HSA-SOH can either form mixed HSA disulfides or be overoxidized irreversibly to sulfinic acid. The role of these modifications in triggering conformational and functional alterations is currently being investigated.
Quiescin/sufhydryl oxidase (QSOX): regulation studies in the vascular system

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QSOX are flavoenzymes that introduce disulfide bonds in a wide range of peptides and proteins in vitro. Since they are an ancient fusion of thioredoxin domains and the FAD-containing protein Erv1/Alr, catalysis takes place through a series of disulfide exchanges, with oxygen as the final electron acceptor. They have been described in chicken egg white, guinea pig endometrium, rat male genital tract, mouse epidermis, human lung fibroblast and neuroblastoma, rat and guinea pig central nervous system. Our interest in QSOX emerged when we were studying redox processes involved in vascular remodeling, particularly the role of critical thiols in modulating the vascular NADPH oxidase activity. Therefore, we first analyzed the presence of QSOX in vascular tissues by immunohistochemistry, which showed its presence predominantly at the extracellular milieu of human and rat arteries, mainly close to microfibrils of elastic fibers. Then, we produced a recombinant mouse QSOX fused to a histidine tag, that was used to obtain hyperimmune sera anti-QSOX. Results from indirect immunofluorescence microscopy showed a perinuclear and granular QSOX expression in rat aortic smooth muscle cells, while RT-PCR assays demonstrated that, in contrast to lung fibroblast, vascular QSOX mRNA was not upregulated by cell confluence or serum depletion-induced quiescence. In addition, QSOX expression in rat smooth muscle cells was not altered by angiotensin II (100 nM), N-acetylcysteine (5 mM) or LPS (10 ng/ml), by immunoblotting experiments, which suggested that it is not under redox control only. Still employing the hyperimmune serum, we analyzed QSOX expression during vascular remodeling after balloon injury in rat carotids. Preliminary results indicated an increase in QSOX immunostaining immediately after the lesion (2h-1day), followed by a decrease and a new increase after 10-15 days in the ipsilateral artery, consistent with a role in apoptosis and tissue remodeling. Interestingly, in some cases, especially 10 days after the lesion, the contralateral QSOX staining seemed to be stronger than that observed in the injured artery, suggesting that it may be responsive to a neurogenic signal related to the balloon injury.
Interaction between Protein Disulfide Isomerase and Vascular NADPH Oxidase Activity

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The oxidative state of cysteine residues is a critical factor determining enzyme activity. However, kinetics of spontaneous thiol-disulfide exchange, based solely on the cell redox status, may be too slow to account for significant target regulation. Thiol oxidoreductases catalyze thiol reactions up to 10^4 times faster and may have an important signaling role. The protein disulfide isomerase (PDI) belongs to this class of enzymes, and has a crucial role in the ER, cytoplasm, and on the cell membrane assisting protein folding and maintaining protein thiols in the functional redox state in accordance to redox environment.

Redox-dependent signal transduction plays a central role in vascular physiology. It relies on reactive species generation systems, such as the vascular isoforms of NADPH oxidase, which are the most important source of superoxide radical in vessels. As recent evidences suggest that the NADPH oxidase may be redox regulated by thiol exchange reactions, we hypothesize that PDI interacts functionally and spatially with the vascular oxidase.

Overexpression studies of fluorescence-tagged fusion constructs of NADPH oxidases in HEK293 cells revealed perfect colocalization of the oxidase proteins Nox1, Nox2 and Nox4 with endogenous PDI. Results were confirmed by co-immunoprecipitation. Moreover, in cultured vascular smooth muscle cells (VSMC), PDI colocalizes and co-precipitates with endogenous Nox and p22^phox subunits of the NADPH oxidase. Nox1- as well as Nox4-dependent radical generation in NADPH oxidase expressing HEK cells was efficiently reduced by bacitracin, a pharmacological inhibitor of PDI as well as by transfection of anti-PDI siRNA. Reactive thiols on the NADPH oxidase are probable targets for PDI activity. Mutation of conserved cysteines of Nox1 and Nox4 identified two critical cysteine residues, which disrupt oxidase activity and interfere with PDI co-localization. More important, in VSMC bacitracin or antisense against PDI also significantly attenuated oxidase activity and radical mediated angiotensin II-induced AKT activation or thrombin-induced MCP-1 secretion.

Therefore, PDI is a novel regulatory protein of vascular smooth muscle cell NADPH oxidases. Furthermore, we demonstrated that Nox function depends on specific conserved cysteines, which may represent the targets for PDI activity.
Peroxiredoxins (Prxs) are abundant thiol-dependent peroxidases that rely on reactive cysteines to decompose peroxides. Prx can be divided into two groups, namely 1-Cys Prx and 2-Cys Prx, depending on the number of cysteine residues involved in catalysis. Recently, several reports showed that Prx are very versatile proteins, being capable to exert also chaperone function (Jang et al., Cell, 2004, 117: 625). In contrast with other proteins with reactive cysteines, Prxs can suffer greater changes in the oxidation state of their reactive cysteine. As an example, this residue can be oxidized to sulfenic acid (R-Cys-SOH), which in most proteins is not reducible by classical reducing agents. Interestingly, sulfenic acids in some Prxs can be specifically reduced by sulfiredoxin in an ATP-dependent process (Biteau et al., Nature, 2003, 425: 980). We have studied Prx from Saccharomyces cerevisiae by structural and functional approaches as well as their patterns of gene expression. As a consequence, we have demonstrated that although all five yeast Prxs have the same biochemical activity (thioredoxin peroxidase), their functions are not completely redundant. In this regard, we have shown that cytosolic thioredoxin peroxidase I (cTPxI/Tsa1/YML028W) is specifically important for the defense of yeast with dysfunctional mitochondria, whereas mitochondrial thioredoxin peroxidase I (mTPxI/PrxI/YBL064C) is more active in respiratory conditions. On the other hand, cytosolic thioredoxin peroxidase II (cTPxII/Tsa2/YDR453C) appears to be an important backup for cTPxI for the defense against organic peroxides, independently of the functional state of mitochondria. Finally, alkylhydroperoxide reductase (Ahp1/cTPxIII/YLR109W) was very resistant to oxidative inactivation and more efficient in the removal of organic peroxides. Interestingly, during these studies, we have discovered a novel aspect of Prx versatility: the peroxidase activity of mTPxI (and also of 1-Cys Prx from other organisms) can also be supported by ascorbate (vitamin C). This ascorbate peroxidase activity is probably related to the fact that sulfenic acid (Cys-SOH) is stabilized in 1-Cys Prxs. Since ascorbate is present in high amounts in cells, the couple (ascorbate/Cys-SOH) can represent unexplored new aspects of redox chemistry in cells, not yet explored. (Supported by FAPESP and CNPq)