Oxidized thiols markedly amplify the vascular response to balloon injury in rabbits through a redox active metal-dependent pathway

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Abstract

Objective: Our aim was to assess whether exposure to oxidized thiols – a known usual consequence of oxidant stress – has the potential to affect the vascular repair response to angioplasty-induced injury. In addition, we also assessed the role of redox active metals in disulfide effects. Methods: In 82 rabbits submitted to overdistention of iliac arteries, the following variables were analyzed: neointimal thickening, immunoreactivity to Proliferating Cell Nuclear Antigen, and cellular and collagen densities. Results: A single intraarterial challenge of oxidized glutathione (GSSG, 6.5 \textmu mol/kg) during and immediately after injury triggered a marked increase of the vascular repair reaction, as follows: (A) at day 7 after injury, there was a 2.7-fold increase in proliferation (\(p<0.001\) vs. control); (B) at day 14, there was increase of intimal/medial area ratio to 1.35\(\pm\)0.14, vs. 0.56\(\pm\)0.08 in controls. Proliferating cells increased to 5.5\(\pm\)0.8 cells/mm\(^2\), vs. 2.2\(\pm\)0.5 in controls (\(p<0.002\) for both variables). Overall cellularity was enhanced 2.2-fold; (C) at day 28, there was ongoing vessel wall proliferation, contrarily to controls. All GSSG effects were completely prevented by co-infusion of reduced glutathione (GSH) and were mimicked by cystine (6.5 \textmu mol/kg). The uninjured artery showed no response to disulfides. To assess the role of redox active metals in GSSG action, the effects of 1,10-phenanthroline or N-CBZ-Pro-Leu-Gly hydroxamic acid (HXA), metal chelators with metalloproteinase inhibitor properties, were evaluated. Both compounds totally blocked the GSSG-induced amplification of vascular responses. In rabbits not exposed to GSSG, HXA decreased neointimal thickening by 50% (\(p<0.05\)). Conclusions: Exposure to excess disulfide levels early after vascular balloon injury markedly amplified the late cellular response through interaction with redox active metals. These pathways can potentially mediate noxious effects of oxidative stress in vessels. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Rabbit models; Angioplasty; Restenosis; Free radicals; Thiols; Metals, redox active; Antioxidants; Metalloproteinases

1. Introduction

Recent observations have uncovered an emerging broad role of ubiquitous redox processes in cellular signal transduction [1–3]. Accordingly, free radicals and related intermediates have been implicated in physiological or pathological control of vascular tone and structural remodeling [1,2]; their signaling role could, therefore, be particularly relevant in the vascular injury repair reaction.

Previous work from our laboratory focused on the effects of reactive oxygen species (ROS), particularly the superoxide radical, on acute vascular response to injury; in a dog model of balloon coronary injury, superoxide dismutase completely prevented acute tonic vasoconstriction [4]. Other experimental data suggest a role for ROS in neointimal thickening [5,6] or vascular remodeling [7] late after arterial injury. A recent clinical trial further supports this notion [8].

Despite those evidences, the redox signaling hypothesis is yet to be further explored. In particular, in addition to provoking a redox imbalance, angioplasty is often per-
formed under circumstances of increased exposure to the consequences of oxidative stress. It is unclear whether redox processes associated with free radical formation, such as sulfhydryl oxidation and interaction with metal complexes, further affect the vascular responses. Thiol-disulfide equilibrium is a direct consequence, as well as a determinant of the normal redox cell defense mechanism [9]. Thiol-disulfide exchange reactions significantly affect many cell functions, having been increasingly implicated in the regulation of enzyme activity, often through irreversible modifications [9]. At the same time, sulfhydryl oxidation is a final common pathway of most oxidative events such as xenobiotic exposure and inflammation [9,10]. Accumulation of disulfides within the vascular wall has been reported in atherosclerosis [11] and hyperhomocysteinemia [11,12] and is very likely to occur in diabetes mellitus [13] and possibly arterial hypertension [3] – conditions of enhanced risk for vascular disease. Therefore, disruption of thiol-disulfide balance by infusions of oxidized glutathione, and ultimately of other disulfide compounds, could be a suitable in vivo model of the consequences of oxidative stress. Indeed, intravenous infusions of oxidized glutathione induced cardiac dilatation accompanied by collagen loss [14,15]. Another open question is whether the initial oxidative stress after injury [4] may have any influence on long-term vascular repair. The aim of the present study was to investigate, in a rabbit model, the effects of transient exposure to disulfide compounds soon after arterial injury on the long-term repair reaction. The vascular response was analyzed through quantification of neointimal thickening and vessel wall proliferation indexes, cellularity and collagen content. In addition, the effector role of redox active metal complexes in those processes was also examined through the use of antagonist compounds.

2. Methods

2.1. Model

This model has been described in detail in previous studies from our laboratory [16]. Briefly, male New Zealand White rabbits weighing 2.6±0.4 kg (range 2.1–3.0, total n=82) were anesthetized with sodium pentobarbital 25 mg per kg i.v. The right femoral artery was dissected and opened 4–5 cm distal to the inguinal ligament. A soft 3-F catheter was advanced retrogradely through the left iliac artery and located 1–2 cm above iliac bifurcation for drug infusions. Regular heparin (Roche Laboratories, Campinas, Brazil) was given as an IV bolus, 250 IU per kg, 10 min before the introduction of a coronary-type balloon angioplasty catheter (from several manufacturers, with balloon length of 20.0 mm and inflated diameter of 3.0 mm), which was advanced retrogradely through the right iliac artery until its origin. Arterial injury was induced by 3 series of balloon inflations (each one 3 times 8.0 atm for 30 s), throughout the entire length of the ilio-femoral artery. This protocol was designed to induce about 30–40% overstretch, as verified by arteriography in 8 other preliminary rabbits. The uninjured left iliac artery served as a control. The right and left femoral access branches were tied; benzathine-penicillin and gentamycin were administered. After survival periods of 7,14 or 28 days, the rabbits were killed with sodium pentobarbital. After in situ perfusion at systemic pressure for 30–40 min, right and left iliofemoral arterial fragments were immersion-fixed in 4% buffered formalin. This study complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and was approved by a scientific/ethics committee from our Institution.

2.2. Treatment groups

Continuous intraarterial infusions of GSSG and of other compounds were performed through an infusion pump (Harvard Apparatus, South Natick, Mass.). All drugs were diluted in phosphate-buffered saline solution, pH 7.4 at room temperature for a final volume of 20 ml, always terminating 150 minutes after angioplasty. The control group received an equal volume of saline solution. No further drugs were administered thereafter. All compounds were from Sigma (St. Louis, Miss.), stored according to their specifications, and prepared immediately prior to use, except for the 1,10-phenanthroline stock solution, which was frozen at −20°C. Cystine was dissolved in small-volume HCl at pH 2.0, which was later adjusted to 7.0.

Oxidative challenge of the injured artery was performed with an infusion of GSSG, 6.5 (4.0 mg) or 65.3 (40.0 mg) μmol per kg body weight, starting immediately (2 min) before angioplasty. To assess the role of a possible redox imbalance upon GSSG effects, some rabbits receiving the 6.5 μmol per kg GSSG dose were concomitantly given an infusion of reduced glutathione (GSH) at the dose of 13.0 μmol per kg (which corresponds to the stoichiometric balance of the equilibrium reaction). To assess the possible role of redox-active metal ions as effectors of vascular oxidative stress, 2 compounds were tested for their ability to block GSSG (6.5 μmol per kg) effects: a) 1,10-phenanthroline (orthophenanthroline, OP), a non-specific cell-permeable divalent metal chelator with a high affinity for zinc [17,18] and a potent well-known metalloproteinase inhibitory effect in vitro [19]; OP was given as a single 50 nmol per kg infusion, starting 10 minutes before angioplasty. This dose was chosen from previous studies from our laboratory (Pedro MA et al., unpublished data) showing its blocking effect on in vivo flow-dependent free radical generation [20]; b) N-Carbobenzyl-oxi (CBZ) Pro-Leu-Gly hydroxamic acid (HXA), a metal chelator with reported inhibitor properties of human collagenase [21] and pos-
sibly of other metalloproteinases, given as a single 9.2 μmol per kg infusion. Previous reports have shown absence of cellular toxicity of OP and hydroxamic acid peptide compounds [18,22–27]. Finally, to assess the role of another disulfide compound, the effects of transient exposure to cystine (6.5 μmol per kg) were also investigated. In summary, according to the type of treatment after angioplasty, the rabbits could be grouped as follows:

1. Control – no treatment (total n=28)
2. GSSG, 65.3 μmol per kg (total n=14)
3. GSSG, 6.5 μmol per kg (n=5)
4. GSSG + GSH (n=5)
5. GSH (n=5)
6. GSSG + OP (n=5)
7. OP (n=5)
8. GSSG + HXA (n=5)
9. HXA (n=5)
10. Cystine, 6.5 μmol per kg (n=5)

2.3. Morphological and immunohistochemical analysis

The injured right iliac artery was cut in a systematic way into 3 equally spaced transverse fragments (4–5 mm in length), sampled in a way to exclude border zones (which could show variable degrees of injury). Another fragment was collected from the uninjured left artery. These fragments were embedded in paraffin and stained with the Verhoeff-van Gieson method, as well as with hematoxylin and eosin. Each fragment was cut into 6–8 samples. The extent of neointimal thickening was calculated with computer-assisted planimetry from manually drawn images of slides projected with a microscopic slide projector (Carl Zeiss, West Germany). This procedure was highly reproducible (<2% variability). Results were expressed as the intimal/medial area ratio, since this variable is unaffected by the basal size of the artery and is less dependent on the fixation procedures; as discussed in Results, analysis of absolute neointimal areas yielded similar results, but with higher variability. The evolution of the repair reaction and the widespread expression of γ- and α-actin by neointimal cells were previously characterized in our laboratory [16] and are analogous to other similar models [28–30], with near-maximal proliferation of an incipient neointima at day 7 after injury. The neointimal thickening is prominent thereafter, so that its size is typically about twice the medial size by day 28 after injury; proliferative activity is markedly decreased by day 14 after injury and already ceased by day 28.

Immunohistochemical staining for Proliferating Cell Nuclear Antigen (PCNA-cyclin) was performed with the PC-10 mouse antibody from Dako Corporation (Santa Barbara, Calif., USA). Careful attention was given to the timing of specimen fixation, kept at 20–26 hours. Arterial fragments were incubated for 16 hours with the primary antibody at a 1:20 dilution at 4°C. The primary antibody was revealed through the avidin-biotin-peroxidase method (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif. USA). The extent of cell proliferation was calculated by manually counting the number of nuclei positively stained for PCNA/cyclin antigen in medial or in intimal layers with the aid of a Sony computerised imaging system. The transverse sections of each arterial fragment were analyzed from at least one field per each quadrant, and results expressed as the average data. Thus, results for each rabbit reflect the data of at least 12 fields/injured segment.

2.4. Quantification of collagen and cellular density

Using the same computerised system described above, collagen density was calculated with the previously described Picrosirius polarization method [31], which is based on the binding of the Syrius Red stain to collagen fibers, leading to their birrefringency under polarized light. Cell nuclei density was manually calculated from the same fields under normal light. Results, therefore, were expressed as percent of total area (for collagen) or number per unit area (for cells). As with histochemical data, each variable was calculated from 4 fields per transverse section.

2.5. Definition of morphometrical indexes

Although the relative collagen or cell densities defined above are the truly independent morphometric variables, they convey little information on the total quantity of collagen or cells, since the neointimal size more than doubled after disulfide exposure. To also obtain an index of those values, the individual collagen and cell densities were multiplied by the respective absolute intimal areas. The results, therefore, were referred to as total collagen area (or mass) (expressed in mm2) or total cell number, each variable expressed as the average value per transverse section. The fractional labelling described in Table 1 was calculated as the number of cells labelled for PCNA/cyclin in relation to total cell number.

2.6. Measurement of plasma glutathione levels

In a separate group of 8 additional rabbits given either saline, GSH (13.0 μmol per kg), GSSG (6.5 μmol per kg) or their combination (2 rabbits each), distal right femoral arterial blood was carefully collected through a short polyethylene catheter before and at different time points after angioplasty. Plasma was quickly separated in a microcentrifuge and stored in liquid N2 until analysis, which was performed through NADPH consumption following reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in acidified samples [32]. GSSG levels were analyzed after treatment of samples with 2-vinylpyridine [32].
Table 1
Fractional labelling indexes for PCNA/cyclin

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Fractional labelling (%)</th>
<th>Number of PCNA/cyclin labelled cells (cells/0.01 mm²)</th>
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<tr>
<td>Control</td>
<td>1.96±0.46</td>
<td>12.4±2.8</td>
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<tr>
<td>GSSG</td>
<td>4.40±0.84*</td>
<td>60.3±12.6*</td>
</tr>
<tr>
<td>GSSG + GSH</td>
<td>2.90±0.48</td>
<td>18.9±3.6#</td>
</tr>
<tr>
<td>GSSG + OP</td>
<td>2.48±0.55</td>
<td>15.6±3.8</td>
</tr>
<tr>
<td>OP</td>
<td>2.74±0.25</td>
<td>15.2±1.8</td>
</tr>
<tr>
<td>GSSG + HXA</td>
<td>4.14±0.44*</td>
<td>16.5±2.4#</td>
</tr>
<tr>
<td>HXA</td>
<td>3.44±0.29</td>
<td>14.9±1.9</td>
</tr>
<tr>
<td>Cystine</td>
<td>4.64±0.56*</td>
<td>91.0±15.8*#</td>
</tr>
</tbody>
</table>

* p<0.05 vs. control; # p<0.05 vs. GSSG. Data are mean ±1 SEM.

2.7. Statistical analysis

Data are shown as mean ±1 S.E.M.. Comparison of the time courses of GSSG responses vs. control group was performed by Student’s t-tests. Other morphometric parameters were analyzed as a single one-way ANOVA and Student-Newman-Keuls multiple range tests at a 0.05 significance level, with The Primer of Biostatistics computer program (version 3.0, 1992, by SA Glantz, McGraw Hill, Inc.).

3. Results

3.1. Effects of GSSG exposure in non-injured iliac arteries

No evident alterations were induced in uninjured left iliac arteries following exposure to GSSG (Fig. 1 and Fig. 2, columns to the left). There was no focal or diffuse increase in neointimal thickening or positivity to PCNA/cyclin, so that the intimal/medial area ratio and cell proliferation indexes remained close to the very low control values. In addition, no alteration was observed on cellularity or collagen content of the medial layer (data not shown). Therefore, placement of the infusion catheter had a negligible influence on vascular responses in this model. It is known that ligature of the femoral branch has only minor effect in proximal iliac flow in this model [20].

3.2. Time course of arterial response to injury in GSSG-exposed rabbits

The time course of neointimal thickening and cell proliferation observed after angioplasty in control arteries was similar to that reported previously and is depicted in Figs. 1 and 2. By day 7 after injury, there was little neointimal thickening, but the few neointimal cells were in near-maximal proliferation. Neointimal growth was prominent at day 14 after injury, and its size almost doubled until day 28, whereas smooth muscle replication was significantly decreased by day 14 and similar to the negligible control rates by day 28. In contrast, rabbits exposed to GSSG at a dose of 65.3 μmol per kg exhibited an average 2.1-fold enhancement of the extent of neointimal thickening (p<0.002 vs. control); this marked enhancement was prominent at day 14 after injury. Since absolute medial areas were similar between these 2 groups (data not shown), results were expressed as the intimal/medial area ratio, as reported before [16]; data expressed as the absolute intimal areas yielded similar results, but with higher variability.

The extent of cell proliferation after injury exhibited a marked and persistent increase after exposure to GSSG (p<0.002 vs. control at 7, 14 and 28 days). At day 14 after injury, there was a 3-fold increase; in addition, in marked contrast with the control group, GSSG-exposed rabbits showed persistent intimal replication at day 28. Thus, there was amplification of vascular responses with exposure to...
65.3 μmol per kg GSSG, as assessed 14 days after injury. A similar amplification was observed after GSSG doses of 6.5 μmol per kg or 0.65 μmol per kg (data not shown, n=3). Since the variability observed with the 6.5 μmol per kg dose was the smallest, this dose was chosen for subsequent studies of cellularity, collagen content and effects of antagonists.

3.3. Plasma glutathione levels

Baseline total glutathione levels (reduced + oxidized) were 135±10 ng/ml and, immediately after angioplasty, were changed to average values of 139, 239, 207 and 305 ng/ml in the rabbits given saline, GSSG, GSH or their combination, respectively. The corresponding values 60 min after injury were 140, 242, 204 and 229 ng/ml. Baseline GSH/GSSG ratio was 1.58±0.34 and remained essentially unchanged in the control group, while changing to average peak values of 0.87, 3.03 and 1.92 after administration of GSSG, GSH or their combination, respectively.

3.4. Effects of GSH co-infusion

The vascular response assessed 14 days after balloon injury in the absence or presence of 6.5 μmol per kg GSSG is depicted in Fig. 3. The intimal/medial area ratio increased from 0.56±0.08 to 1.35±0.14 after exposure to GSSG (p<0.001); this increase was totally prevented by concomitant administration of GSH, whereas GSH alone had no effect. Similar findings were observed for cell proliferation; again, GSSG exposure induced a significant increase vs. control in the number of replicating cells (5.5±0.8 vs. 2.2±0.5 cells/0.01 mm2, respectively, p<0.01), an effect completely prevented by GSH, which had no effect by itself. A similar and significant increase in both neointimal thickening and cell proliferation also occurred after exposure to cystine. In order to further characterize the composition of the enhanced neointima, cellularity and collagen content were analyzed; such results are depicted in Fig. 4 and Fig. 5. In the neointimal layer, total cell number was more than doubled after exposure to GSSG and more than 3-fold increased after cystine. However, the increase in neointimal cell number occurred in the same proportion as the non-nuclear material, so that cell density was essentially maintained. The cellular effects of GSSG were totally prevented by the concomitant administration of GSH, which had little effect by itself. The density of collagen was significantly decreased after disulfide exposure (by approximately 50% with GSSG and 25% with cystine). On the other hand, the total neointimal collagen mass was maintained after GSSG, but significantly increased after cystine (Fig. 4, lower panel). Fig. 5 (panels A, B and C) shows a representative example of this decrease in collagen density associated with GSSG exposure and illustrates the uniformly observed finding that such decrease was more pronounced at the intimal region adjacent to the medial layer. In contrast, the increased indexes of cell proliferation were generally observed at the lumen-adjacent neointima. Concomitant administration of GSH significantly prevented GSSG-induced loss in neointimal collagen density (Fig. 4, lower panel).

With respect to the medial layer, the alterations induced by GSSG paralleled those found in the intimal layer. The cell density exhibited a 26% decrease in injured arteries, as...
3.5. Effects of OP or HXA on GSSG-induced enhancement of vascular response

Administration of either OP or HXA markedly and completely prevented the GSSG-induced amplification of vascular response to angioplasty (Fig. 6 and Fig. 5, Panel D). This effect was evidenced by a profound reduction in neointimal thickening, which reached values even below those observed in control rabbits after injury. The number of proliferating cells was also reduced toward control values, although to a lesser extent. Fig. 6 also shows that, even for rabbits not exposed to GSSG, HXA administration decreased intimal/medial ratio to 0.24±0.04, vs. 0.56±0.08 in controls (p<0.05). Total cell number was also decreased by 60 (Fig. 7). The amount of proliferating cells, however, was not decreased in relation to controls (Fig. 6).

Fig. 7 (upper panel) shows that OP or HXA had a significant effect on the antagonism of GSSG-induced increase in total cell number. Fig. 7 (lower panel) shows that the decrease in collagen density after GSSG was effectively curtailed after OP or HXA, whereas total collagen content was essentially unaltered. In addition, the increase in cellularity and decrease in collagen density observed in the medial layer after GSSG were completely prevented by each compound (data not shown).

3.6. PCNA/cyclin fractional labelling

Table 1 depicts the data concerning the fractional labeling of PCNA/cyclin and the total number of labeled cells. Exposure to disulfides induced a significant increase of both indexes. The effects of GSSG on the number of labelled cells were totally prevented by concomitant GSH administration, as well as by administration of OP or HXA. On the other hand, the increase in fractional labelling by GSSG or cystine was only partially abrogated by co-infusion of GSH or OP; such increase was not prevented by treatment with HXA.

4. Discussion

The present study showed that a single infusion of GSSG at the time of angioplasty induced a marked increase in neointimal thickening and vessel wall proliferation indexes in the injured, but not in the uninjured rabbit iliac artery. The effects of GSSG were completely prevented by co-infusion of reduced glutathione and were shared by cystine, indicating that they result from imbalance of the thiol-disulfide redox equilibrium. In addition, all GSSG effects were totally prevented by OP or HXA, indicating that metal complexes are crucially involved as effector mechanisms of the disulfide exposure.

Fig. 4. Upper Panel: Bar graph analogous to Fig. 3 depicting cellular density (open bars, quantified as nuclei per unit area) and the total cellularity index (dark filled bars, see section 2 for definition). There was significant increase in total cellularity after disulfide administration (6.5 μmol per kg). Concomitant infusion of GSH (13.0 μmol per kg) completely abrogated GSSG effects, whereas GSH alone induced no change vs. control. Cellular density, on the other hand, was not significantly modified by disulfide exposure. Lower Panel: Effects of GSSG or cystine exposure (6.5 μmol per kg) on the density of collagen fibers (open bars) or total collagen content (dark filled bars, see Methods for definition). Both disulfide compounds significantly decreased collagen density, although the total collagen mass was unaffected by GSSG and increased after cystine (p<0.05). The alterations induced by GSSG were completely prevented by concomitant administration of GSH (13.0 μmol per kg), which had little effect by itself. * p<0.05 vs. control group and † p<0.05 vs. GSSG-exposed group; n=5 rabbits/group.

compared with the non-injured control left artery; however, the medial area increased by 25% after injury (the increase was similar with or without GSSG), so that the overall cellularity was essentially maintained (data not shown). After injury, collagen density significantly decreased following GSSG exposure in relation to controls (respective average values of 14 and 27% area collagen, p<0.01). Such effects were significantly prevented by GSH.
Fig. 5. Histological sections of rabbit iliac arteries stained with the Verhoeff-van Gieson method under normal light (left column) and the corresponding fragment stained with Syrius Red plus hematoxylin under polarized light (right column). The arterial lumen is at the upper right corner of each picture and the white arrows denote the internal elastic laminae; M=medial layer. Panel A, Uninjured iliac artery. Panel B, Control rabbit 14 days after angioplasty, showing neointimal thickening, fracture of internal elastic lamina and neointimal collagen accumulation. Panel C, GSSG-exposed rabbit (6.5 μmol per kg), showing increased neointimal thickening with reduced collagen density, which was more pronounced at the region adjacent to the media; the collagen density in the medial layer was also decreased. Panel D, GSSG-exposed rabbit treated with HXA (9.2 μmol per kg), showing important decrease in neointimal thickening and a relative preservation of neointimal collagen framework. 40x.
Fig. 6. Bar graph depicting the effects of 1,10-phenanthroline (OP, 50.0 nmol per kg) and N-(CBZ) Pro-Leu-Gly hydroxamic acid (HXA, 9.2 μmol per kg) on the amplification of neointimal thickening (open bars) and cell proliferation (dark filled bars) induced by 6.5 μmol per kg GSSG at day 14 after angioplasty. For clarity, data for control and GSSG infusion are reproduced from Fig. 3. The marked increase in both variables induced by GSSG was completely prevented by OP or HXA. In the absence of GSSG, HXA alone led to a significant decrease in neointimal thickening, though not of cell proliferation, vs. control. * p<0.05 vs. control rabbits and ‡ p<0.05 vs. GSSG-exposed group; n=5 rabbits/group.

4.1. Modelling considerations

Our study focused on cell proliferation and neointimal thickening as the expression of vascular repair, which is in line with the proliferative paradigm of post-angioplasty restenosis [29,30]. However, the role of vascular proliferation in restenosis is controversial [30]. The present study reproduced a marked and sustained increase in cell proliferation rates, with an enlarged neointima at the 2nd week after injury. Interestingly, though, the expected massive neointimal increase at the 4th week after injury was not observed. This phenomenon is opposite to the accelerated ‘catch-up’ thickening of the neointima after an initial inhibition described previously after administration of a metalloproteinase inhibitor [26] and may be due to increase in the rates of cell death, as discussed below. The reliability of PCNA-cyclin as a proliferation index has been questioned [30]. However, PCNA indexes after arterial injury are similar to those of other markers [33]. In addition, the labelling index in the present study was well within the range previously reported in the rabbit model with bromodeoxyuridine [34]. Meticulous attention was given to the timing of specimen fixation, a known factor affecting PCNA labelling [35]. These considerations and the fact that ours was a comparative study indicate that the labelling method had negligible influence on our results.

4.2. Factors influencing neointimal thickening after exposure to GSSG

In addition to cell proliferation, other processes that modulate neointimal thickening may have played a role in GSSG effects. First, as discussed above, increased rates of cell loss may have prevented a sustained increase in neointimal size despite a marked and persistent increase in proliferation with GSSG (Figs. 1 and 2). In fact, apoptotic cell loss has been demonstrated to modulate neointimal size after injury [36]. Second, the increase in neointimal thickening could not be attributed only to greater cellularity, since cell density was maintained, suggesting that there was a proportional increase in extracellular matrix. Collagen density was decreased by 50%, though, which is consistent either with accumulation of non-collagenous
matrix or with loosening of the matrix. Therefore, in addition to cell proliferation, alterations in extracellular matrix may have been equally important in disulfide effects. This is in line with the hypothesis of metalloprotease activation discussed below, although the decreased collagen density might be due not only to its enhanced degradation but also to impaired synthesis. Such impaired synthesis is possible, considering that enhanced GSSG-induced cell proliferation was probably accompanied by delayed differentiation, and collagen accumulation is normally a feature of later stages of the vessel repair reaction [29,30]. Third, GSSG could have increased smooth muscle cell migration [37]. Signal transduction of platelet-derived growth factor – a key mediator of cell migration [37] – critically requires hydrogen peroxide [38]. Also, metalloprotease activation, a redox-sensitive pathway [19], is an important step for migration [25,26,39]. The marked reduction on the extent of neointimal thickening by HXA or OP, which can inhibit metalloproteinases, together with less reduction in proliferation indexes and lack of effect on fractional labelling (Table 1) is consistent with a role of metals in cell migration. Finally, the contribution of blood-borne cytokines and growth factors evoked by circulating disulfides in other organs cannot be excluded.

4.3. Redox mechanisms involved in GSSG effects

An implication suggested by our results is that thiol oxidation and consequent disulfide accumulation, which are well-known consequences of an oxidative insult, may be deleterious by themselves, acting as an amplifying effector component of the oxidative cascade. The redox pathways involved in disulfide effects may involve a complex host of reactions, including not only thiol exchange [9,10], but also accumulation of hydrogen peroxide due to consumption of cellular reducing power (e.g., NAD(P)H-dependent), decrease in the levels of nitrosothiol compounds [40], formation of the thyl radical (RS) [41] and finally superoxide formation from strongly reducing disulfide radical anions (RSSR⁻) [10,41]. The latter possibility is less likely, considering that arterial rings exposed to GSSG were reported to exhibit normal endo-

<table>
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<tr>
<th>Intracellular</th>
<th>Reference</th>
<th>Extracellular</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Tyrosine kinases/phosphatases</td>
<td>[2,44]</td>
<td>Ion channels</td>
<td>[45–47]</td>
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<td>Cation channels</td>
<td>[45–47]</td>
<td>Fibroblast growth factor release</td>
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<td>Endoplasmic reticulum Ca²⁺-ATPase</td>
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<td>NF-κB and AP-1 transcription factors</td>
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reduced form in intact cells [9,10,32], whereas oxidative insults such as atherosclerosis increase basal disulfide levels up to 10-fold [11], mimicking the situation modelled in our study.

4.4. The role of metal complexes in GSSG effects

The crucial role of redox active metals in disulfide effects was suggested by their total and complete inhibition by OP and HXA. The simplest explanation for this effect would be a Fenton-like mechanism involving release of metals by injury [56] plus disulfide-induced decrease in hydrogen peroxide removal. An attractive alternative hypothesis could be the interaction between disulfides and constitutive vascular metalloproteinases. It is known that activation of pro-metalloproteinases can be triggered by redox-cycling or oxidant compounds [19,57] and, in particular, by GSSG and other disulfides – which undergo an exchange reaction with the critical cysteine residue bound to the zinc atom at the active site [19]. Hydroxamic acid derivatives were previously shown to inhibit metalloproteinase-dependent smooth muscle cell migration in vitro [25] and in vivo [26]. Yet, this hypothesis should be viewed as speculative, given the lack of specificity of chelator compounds. Non-specific effects of OP, e.g., can arise due to iron, copper, and maybe calcium chelation [18]. Although HXA was designed as a collagenase inhibitor [21], non-specific effects may be anticipated through the wide reactivity of its hydroxamate moiety, not only toward metals, but also, e.g., to peroxynitrite [58]. Finally, one cannot fully exclude that the effect of chelators could be due to arrest in cell division due to profound iron depletion.

The significant effects of these redox-active metal antagonists, whether or not related to metalloproteinases, may implicate an important role for redox active metals in the pathogenesis of conditions associated with elevated disulfide levels, such as hyperhomocysteinemia, diabetes, and atherosclerosis itself [3,4,11–13]. Previous reports showed inhibition of cell proliferation by deferoxamine [59] or synthetic metalloproteinase inhibitors [27]; this is in line with our findings that HXA significantly inhibited neointimal thickening and cellularity even in the control group not exposed to GSSG. These data suggest that metal ions may affect the normal vascular response to injury.

5. Conclusions

The marked effects of disulfide exposure observed in the present study suggest that disulfide accumulation, a known consequence of oxidative stress, has the potential to exert a pathological role in vascular response to injury. Our data also indicate that even a transient early redox imbalance can have profound sustained consequences in late cellular events. These cellular events may include proliferation as well as migration and death. The consequences of disulfide exposure are crucially dependent on the interaction with metal targets, suggesting that redox active metals affect the injury repair reaction. In a broader sense, the present data add to other reports that delineate the existence and importance of a vascular redox signaling network [1,3]. [43–48,50–52]

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