THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 34, pp. 26135–26148, August 20, 2010

Postischemic Deactivation of Cardiac Aldose Reductase

*ROLE OF GLUTATHIONE S-TRANSFERASE P AND GLUTAREDOXIN IN REGENERATION OF REDUCED THIOLS FROM SULFENIC ACIDS**

Received for publication, May 19, 2010, and in revised form, June 7, 2010 Published, JBC Papers in Press, June 10, 2010, DOI 10.1074/jbc.M110.146423

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Aldose reductase (AR) is a multifunctional enzyme that catalyzes the reduction of glucose and lipid peroxidation-derived aldehydes. During myocardial ischemia, the activity of AR is increased due to the oxidation of its cysteine residues to sulfenic acids. It is not known, however, whether the activated, sulfenic form of the protein (AR-SOH) is converted back to its reduced, unactivated state (AR-SH). We report here that in perfused mouse hearts activation of AR during 15 min of global ischemia is completely reversed by 30 min of reperfusion. During reperfusion, AR-SOH was converted to a mixed disulfide (AR-SSG). Deactivation of AR and the appearance of AR-SSG during reperfusion were delayed in hearts of mice lacking glutathione *S*-transferase P (GSTP). *In vitro*, GSTP accelerated glutathiolation and inactivation of AR-SOH. Reduction of AR-SSG to AR-SH was facilitated by glutaredoxin (GRX). Ischemic activation of AR was increased in GRX-null hearts but was attenuated in the hearts of cardiospecific GRX transgenic mice. Incubation of AR-SSG with GRX led to the regeneration of the reduced form of the enzyme. In ischemic cardiospecific AR transgenic hearts, AR was co-immunoprecipitated with GSTP, whereas in reperfused hearts, the association of AR with GRX was increased. These findings suggest that upon reperfusion of the ischemic heart AR-SOH is converted to AR-SSG via GSTP-assisted glutathiolation. AR-SSG is then reduced by GRX to AR-SH. Sequential catalysis by GSTP and GRX may be a general redox switching mechanism that regulates the reduction of protein sulfenic acids to cysteines.

Myocardial ischemia-reperfusion results in the induction of oxidative stress. Ischemia increases the generation of free radicals and reactive oxygen species (ROS),[2] and the generation of these species is increased further upon reperfusion. In several, but not all, models of ischemia-reperfusion injury, redox changes correlate with functional impairment (1–6). The role of free radicals in inducing myocardial injury was supported by early studies showing that treatment with enzymatic and non-enzymatic antioxidants decreased ischemia-reperfusion injury; however, results with exogenous chemicals or enzymatic antioxidants have not been universally positive. In contrast, newer molecular studies with transgenic and gene-deleted animals provide more rigorous and compelling evidence that oxidative stress is a significant component of ischemia-reperfusion injury. Nonetheless, the mechanisms by which ROS induce ischemia-reperfusion injury remain poorly understood.

Although in earlier studies it was assumed that an increase in ROS generation is universally detrimental, more recent work shows that in contrast to their injurious effects at high concentrations ROS at low concentrations can stimulate signaling pathways. Some of these signal transduction events stimulate adaptation and increase tissue resistance to oxidative stress. For instance, it has been shown that brief bouts of ischemia that trigger myocardial preconditioning generate oxidative stress and that antioxidant interventions abolish the cardioprotective effects of ischemic preconditioning (7). Furthermore, in addition to ROS, reactive nitrogen species are also generated in the heart during ischemia-reperfusion. Several studies have shown that the generation of nitric oxide or other reactive nitrogen species during ischemia is essential for the development of a cardioprotective phenotype (8). Nevertheless, the cardioprotective mechanisms of reactive nitrogen species, like those of ROS, are unclear.

Previous work shows that redox changes during myocardial ischemia result in the activation of the enzyme aldose reductase (AR) (9). AR is a multifunctional oxidoreductase that catalyzes the reduction of glucose to sorbitol. The enzyme also reduces a wide range of cytotoxic and bioactive aldehydes generated from lipid peroxidation such as 4-hydroxy-*trans*-2-nonenal, acrolein, and 1-palmitoyl-2-oxovaleryl phosphocholine (10) as well as the glutathione conjugates of unsaturated aldehydes such as 4-hydroxy-*trans*-2-nonenal and acrolein (11). These properties suggest that the enzyme protects the heart from cytotoxic effects of reactive aldehydes generated by oxidizing lipids. In accordance with this view, we have found that inhibition of AR increases the accumulation of lipid peroxidation products in the ischemic heart (12). Inhibition of AR was also found to abolish the cardioprotective effects of late preconditioning in rabbits (12) and increase myocardial infarct size in rats (13).

* This work was supported, in whole or in part, by National Institutes of Health Grants HL55477, HL59378, HL78825, HL89380, HL893802S1, and RR024489. This work was also supported by a postdoctoral fellowship from the America Heart Association (to K. W.).

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[2] The abbreviations used are: ROS, reactive oxygen species; AR, aldose reductase; DD, dimedone; GSH, reduced glutathione; GSTP, glutathione *S*-transferase P; GRX, glutaredoxin; PSSG, protein-SSG; $ONOO^-$, peroxynitrite; TG, transgenic; ESI, electrospray ionization.

Our studies indicate that AR is kinetically activated in the ischemic heart and that this activation is mediated in part by nitric oxide generated by endothelial nitric-oxide synthase (13). In the ischemic heart, nitric oxide reacts with superoxide to form peroxynitrite ($ONOO^-$), which in turn oxidizes AR cysteines (AR-SH) to sulfenic acids (AR-SOH). Oxidation of AR-SH to AR-SOH increases the catalytic activity of the protein while decreasing its sensitivity to inhibition by sorbinil. In addition, we have found that AR could also be activated when endothelial nitric-oxide synthase is stimulated by bradykinin or insulin via a phosphatidylinositol 3-kinase/Akt-dependent mechanism. The increase in AR activity by bradykinin and insulin was found to be accompanied by the selective oxidation of Cys-298, which is located at the active site of the enzyme. Collectively, these observations suggest that AR is activated by nitric oxide under both physiological and pathological conditions. Nevertheless, the fate of the activated enzyme remains unknown. In particular, it is unclear whether AR once activated is converted back to its unactivated form and, if so, which mechanisms mediate deactivation of the enzyme.

In most cells, oxidative modification results in rapid removal of proteins via proteasomal or lysosomal degradation. In principle, oxidized proteins can be converted back to their reduced form; however, such reversibility of modification has not been reported for protein sulfenic acids, and it is currently believed that oxidation of protein thiols to sulfenic acid is a relatively irreversible step and, therefore, of little regulatory significance.

The current study was designed to examine the fate of activated AR in hearts subjected to ischemia-reperfusion. Our results indicate that activation of AR in the ischemic heart is reversed by reperfusion. During this process, AR-SOH, which is generated in the ischemic heart, is converted to a mixed disulfide (AR-SSG) via glutathione *S*-transferase P (GSTP)-catalyzed glutathiolation. The glutathiolated protein is then reduced by glutaredoxin (GRX) to its original, unactivated form. These observations reveal a novel mechanism in which protein sulfenic acids are enzymatically converted into reduced thiols.

EXPERIMENTAL PROCEDURES

Materials—DL-Glyceraldehyde, dimedone, DTT, glycine, peroxynitrite, and reduced glutathione were obtained from Calbiochem and Sigma-Aldrich. Sorbinil (d_6-fluorospiro-(chroman-4,4′-(imidazolidine)-2′,5′-dione) was a gift from Pfizer. Biotin-labeled dimedone was synthesized as published (14). Sephadex G-25 columns, enhanced chemiluminescence (ECL Plus) reagents, horseradish peroxidase-linked streptavidin, and secondary anti-mouse and anti-rabbit antibodies were from Cell Signaling Technology and Amersham Biosciences. All other reagents were of analytical grade.

Animals—Adult male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). GSTP1/P2-null mice were generated on a 129_MF1 background using homologous recombination as described previously (15). GSTP-null mice were bred onto a BL/6 background for six generations before phenotypic characterization. Mice heterozygous for the targeted locus (F1) were backcrossed, and GSTP(+/+), GSTP (+/−), and GSTP(−/−) lines were established. GSTP-null and WT littermates were obtained from Drs. C. Henderson and R. Wolf (University of Dundee) and maintained as independent lines for use in this study (16). Similarly, GRX-TG, GRX-null, and matching WT mice were all backcrossed on a B/6 background, and littermates were used as appropriate controls (17). Mice were treated in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health. Treatment protocols were approved by the University of Louisville Institutional Animal Care and Use Committee.

Global Ischemia-Reperfusion ex Vivo—For global ischemia, hearts excised from male mice (8–12 weeks of age) were cannulated and perfused in the Langendorff retrograde mode as described before (9). Briefly, the hearts were perfused at a constant perfusion pressure of 80 mm Hg with Krebs-Henseleit solution, pH 7.4, containing 118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.25 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 0.5 mM EDTA, and 10 mM glucose and equilibrated with a mixture of 95% O_2 and 5% CO_2 for 10 min. A food grade plastic wrap, fluid-filled balloon was placed in the left ventricle through an incision in the left atrial appendage and inflated to a pressure of 5–8 mm Hg. The perfusion flow rate was between 1 and 2 ml/min for all heart preparations. The hearts were either perfused for 45 or 75 min with Krebs-Henseleit solution under aerobic conditions or subjected to the indicated durations of ischemia alone or ischemia followed by reperfusion. At the end of the protocol, the hearts were freeze-clamped and used for measuring AR activity. The procedure for measuring infarct size has been described before (18).

Measurement of AR Activity—The catalytic activity of AR was measured as described previously (9). Briefly, mouse hearts were homogenized in 10 mM potassium phosphate, pH 7.0, containing 1 mM EDTA, 5 mM DTT, and protease inhibitor mixture (1:100, v/v). Where indicated, the enzyme was reduced with 100 mM DTT for 1 h at 37 °C in 100 mM Tris-Cl, pH 8.0. AR activity was measured in 100 mM potassium phosphate, pH 6.0, containing 0.15 mM NADPH and 10 mM DL-glyceraldehyde. To examine the extent of activation, AR activity was measured in the presence of sorbinil. Our previous studies (13) show that 1 μM sorbinil inhibits AR-SH by 90% and AR-SOH by 20%. Thus, the extent of AR-SOH formation in hearts subjected to ischemia-reperfusion was estimated from percent inhibition of AR by sorbinil using the equation

$$0.9(1 - x) + 0.2x = f \qquad \text{(Eq. 1)}$$

where f is the measured fractional inhibition and x is the fraction of AR in the AR-SOH form. The predicted change in enzyme activity due to AR-SOH formation was then calculated by assuming that the activity of AR-SOH is 4-fold higher than AR-SH activity (13). The predicted value was compared with the experimentally measured enzyme activation during ischemia-reperfusion. In cases when the predicted value (A_p) turned out higher than the measured value (A_{exp}), the difference was attributed to the presence of the inactive glutathiolated form of the enzyme (AR-SSG). The ratio of experimental to predicted activities was then used to estimate the percentage of AR in the catalytically active and glutathiolated forms. The final fraction of each form was calculated using the following set of equations.

$$f_{SH} = (1 - x)A_{exp}/A_p \quad \text{(Eq. 2)}$$

$$f_{SOH} = xA_{exp}/A_p \quad \text{(Eq. 3)}$$

$$f_{SSG} = 1 - A_{exp}/A_p \quad \text{(Eq. 4)}$$

Purification of Recombinant AR, GRX, and GSTP—Human recombinant AR, GRX, and GSTP were purified from *Escherichia coli* on a Ni^{2+} affinity column as described before (19). To examine the molecular mechanism of peroxynitrite-mediated AR activation, reduced DTT-free enzyme (0.3 mg) was incubated with 0.1–1 mM peroxynitrite for 1 h at 4 °C in 10 mM HEPES, pH 7.0, in the dark. The reaction was stopped by desalting the enzyme on a Sephadex G-25 column equilibrated with 10 mM HEPES, pH 7.0, or with 10 mM ammonium acetate, pH 7.0. To document AR-SOH formation, the modified protein was incubated for 30 min with 0.5 mM dimedone (DD), a sulfenic acid-specific reagent (9).

Electrospray Ionization Mass Spectrometry (ESI/MS)—For ESI/MS analysis, the protein was desalted on a Sephadex G-25 column equilibrated with N_2-saturated ammonium acetate (10 mM, pH 7.0). The desalted protein was diluted with the flow injection solvent (acetonitrile:H_2O:formic acid, 50:50:1, v/v/v). The mixture was injected into a Micromass ZMD spectrometer at a rate of 10 μl/min. The operating parameters were as follows: capillary voltage, 3.1 kV; cone voltage, 27 V; extractor voltage, 4 V; source block temperature, 100 °C; and desolvation temperature, 200 °C. Spectra were acquired at the rate of 200 atomic mass units over the range of 20–2000 atomic mass units. The instrument was calibrated with myoglobin (0.15 mg/ml) dissolved in a mixture of acetonitrile and H_2O (50:50, v/v) containing 0.2% (v/v) formic acid.

Two-dimensional Gel Electrophoresis and MALDI-TOF Analysis—Mouse hearts (0.1–0.2g) were homogenized in 4 volumes of protein extraction buffer (20 mM Tris-HCl, pH 6.8) containing 2 mM EDTA, 20 mM *N*-ethylmaleimide, and protease inhibitor mixture (1:100, v/v). Proteins in the sample were separated by two-dimensional gel electrophoresis as described before (9). The proteins were transferred onto polyvinylidene difluoride membranes and probed with polyclonal anti-AR or anti-protein-SSG (PSSG) antibodies. Parallel gels were silver-stained. Spots corresponding to a molecular mass of 36 kDa and pI of ~6.28 were excised and used for MALDI-TOF/MS analysis. The extracted peptides were co-crystallized with a matrix of cyano-4-hydroxycinnamic acid, and a TofSpec 2E mass spectrometer (Micromass) was used to acquire data. A standard mixture of cytochrome *c*, myoglobin, and trypsinogen was used for mass axis calibration. Accelerating potential was set to 20 kV with delayed extraction, and data were collected at a rate of 2 GHz by scanning from 500 to 3000 atomic mass units in the positive ion reflector mode. Average data, represented as a centroid peak, were compared with a battery of databases with 50–100-ppm resolution precision. Peptide *m*/*z* values were analyzed *in silico* for suspect modifications using the Mascot database.

Western Blot Analysis—Heart tissue was homogenized in 10 mM HEPES, pH 7.2, containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM NaF, 10 mM Na_3VO_4, 1% Nonidet P-40, 0.1% SDS, and protease and phosphatase inhibitor mixture (1:100). Proteins were separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. Western blots were developed using ECL Plus reagent (Amersham Biosciences) and detected with a Typhoon 9400 variable mode imager (Amersham Biosciences). Band intensity was quantified by using ImageQuant TL software (Amersham Biosciences), and bands were normalized to actin or Amido Black staining where appropriate. For quantification, individual bands were selected, and the background intensity was subtracted from the band intensity using rolling ball averaging. Western blots shown are representative of three similar gels.

Statistical Analysis—In all cases, best fits to the data were chosen on the basis of the standard error of the fitted parameters. All data are expressed as mean ± S.D. and were analyzed by one-way analysis of variance for multiple comparisons or by Student's *t* test for unpaired data. Statistical significance was accepted at $p < 0.05$.

RESULTS

Activation and Deactivation of AR during Ischemia-Reperfusion—Our previous study showed that the activity of AR is increased in rat hearts subjected to ischemia (9). To examine whether AR is also activated in mouse heart, hearts from adult male C57BL/6 mice were isolated and perfused in the Langendorff mode for 75 min or subjected to 15 min of ischemia followed by 15, 30, and 60 min of reperfusion. By triphenyltetrazolium chloride staining, the infarct size of hearts subjected to 15 min of ischemia followed by 30 min of reperfusion was 3.5 ± 1.6% ($n = 4$), indicating that there was no significant tissue injury or cell death. After the ischemia-reperfusion protocol, the hearts were snap frozen, and the AR activity was measured in the absence and presence of sorbinil. As shown in Fig. 1*A*, in hearts subjected to 15 min of global ischemia, the catalytic activity of AR was increased nearly 2-fold. Moreover, when the enzyme activity was assayed in the presence of 1 μM sorbinil, only 70% of the enzyme was inhibited *versus* 90% inhibition in lysates from hearts subjected to perfusion alone.

The increase in AR activity during ischemia was rapidly reversible. Reperfusion for 15 min resulted in a significant decrease in enzyme activity. There was a progressive decrease in enzyme activity when the duration of reperfusion was increased to 30 or 60 min. The levels of AR activity in reperfused hearts were not statistically different from those in the perfused aerobic hearts. Nonetheless, the return of sorbinil sensitivity, which is a more sensitive measure of enzyme activation, was more gradual. Reperfusion for >30 min was required to fully restore sorbinil sensitivity of the enzyme. These results indicate that as reported for the rat heart (9) brief episodes of ischemia result in AR activation in the mouse heart (albeit with a slightly different time course than in the rat heart) and that reperfusion results in complete recovery of the enzyme from activation (deactivation).

To examine how ischemia and reperfusion affect AR thiols, hearts were subjected to either 15 min of ischemia alone or 15 min of ischemia followed by 30 min of reperfusion. Lysates prepared from these hearts were separated by two-dimensional gel electrophoresis, and the AR was identified by Western blotting. Spots corresponding to the AR-immunoreactive area in a parallel gel

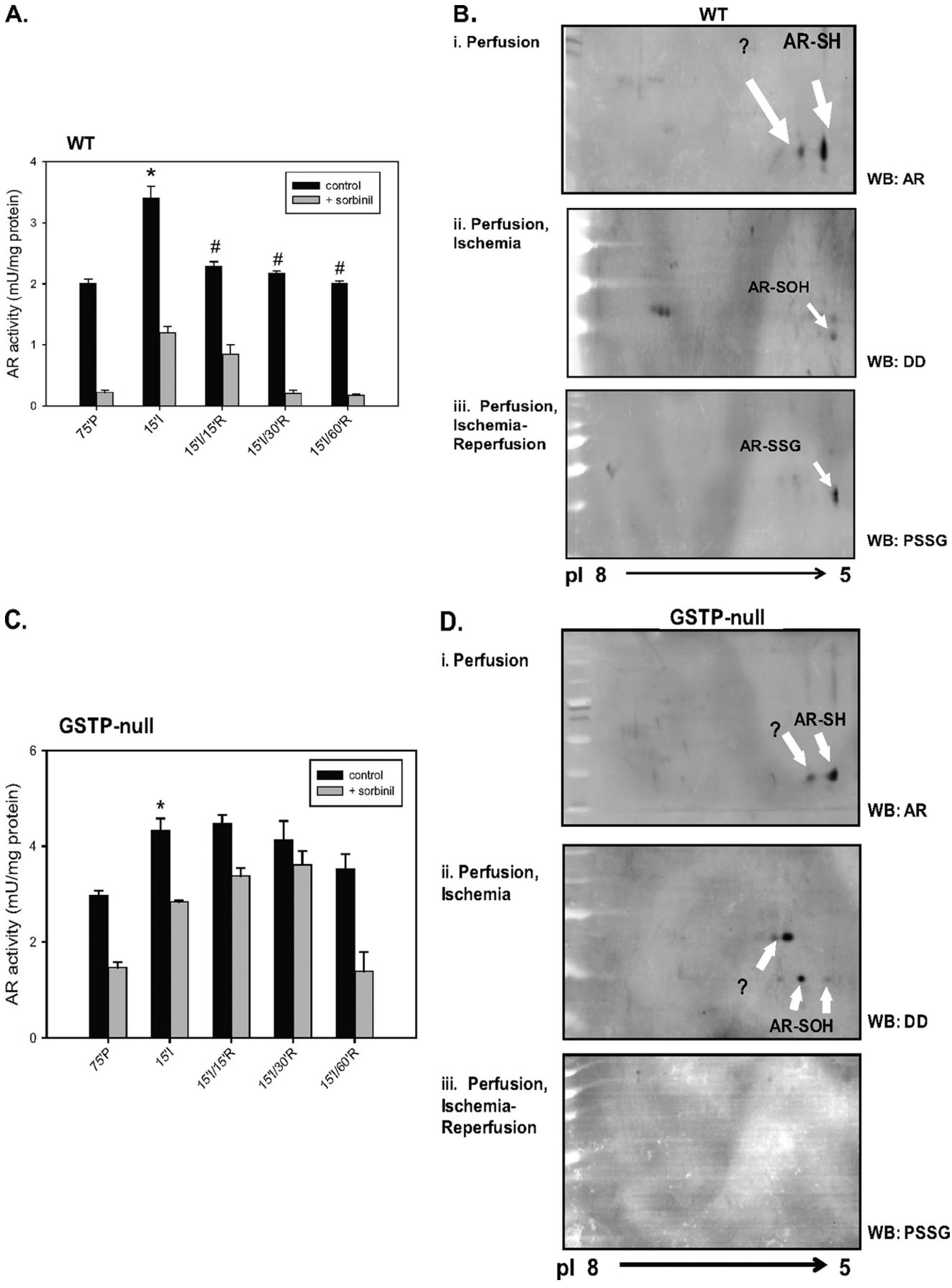

A.
WT
AR activity (mU/mg protein)
control
+ sorbinil
75'P
15'I
15'I/15'R
15'I/30'R
15'I/60'R
B.
WT
i. Perfusion
AR-SH
WB: AR
ii. Perfusion, Ischemia
AR-SOH
WB: DD
iii. Perfusion, Ischemia-Reperfusion
AR-SSG
WB: PSSG
pI 8
5
C.
GSTP-null
AR activity (mU/mg protein)
control
+ sorbinil
75'P
15'I
15'I/15'R
15'I/30'R
15'I/60'R
D.
GSTP-null
i. Perfusion
AR-SH
WB: AR
ii. Perfusion, Ischemia
AR-SOH
WB: DD
iii. Perfusion, Ischemia-Reperfusion
WB: PSSG
pI 8
5

TABLE 1

Post-translational modification of aldose reductase during ischemia-reperfusion

Hearts from WT (A) and GSTP-null (B) mice were subjected to 15 min of global ischemia followed by 30 min of reperfusion. Cytosolic proteins were resolved by two-dimensional gel analysis. Spots corresponding to AR (36 kDa and pI ~ 6.28) were excised and digested, and fragments were analyzed by MALDI-TOF/MS analysis. Post-translational modification of the peptides was determined using Mascot and NCBI databases. Periods in sequences indicate cleavage sites.

A) WT

Residue	Mass (Da)	Sequence	Modification
42-53	1504.6833	R.HIDCAQVYQNEK.E	Carbamidomethyl(C)
79-90	1551.7288	K.LWCTFHDKSMVK.G	Carbamidomethyl(C)
170-177	882.5634	R.ILNKPGLK.Y	
178-195	2218.1121	K.YKPAVNQIECHPYLTQEK.L	Glutathione (C)
204-233	3262.6895	K.GIVVTAYSPLGSPDRPWAKPEDPSLLEDPR.I	
244-251	901.5599	K.TTAQVLIR.F	
252-256	660.4156	R.FPIQR.N	
298-308	1279.6250	R.VCALMSCAKHK.D	Glutathione (C);Oxidation (C)

B) GSTP -null

Residue	Mass (Da)	Sequence	Modification
42-53	1504.6833	R.HIDCAQVYQNEK.E	Carbamidomethyl(C)
79-90	1551.7288	K.LWCTFHDKSMVK.G	Carbamidomethyl(C)
178-195	2218.1121	K.YKPAVNQIECHPYLTQEK.L	
196-203	112.5120	K.LIEYCHCK.G	2 Carbamidomethyl(C)
276-294	2280.1350	K.VFDFELSNEDMATLLSYNR.N	Oxidation
298-308	1279.6250	R.VCALMSCAKHK.D	2 Oxidation (C);Oxidation (M)

were excised and subjected to MALDI-TOF analysis. In hearts subjected to perfusion alone, AR was identified as a major spot (indicated as AR-SH in Fig. 1*B*, *i*) corresponding to the reduced enzyme. A minor AR form (indicated as "?") was also observed. The nature of the spot is not clear, but it is unlikely to be another thiol-modified form of the enzyme because it was also observed when the reduced enzyme was tested. It might represent an incompletely alkylated form of the protein. In two-dimensional gels of lysates from the ischemic heart, AR migrated to a different position than that in the perfused hearts. To determine whether these were due to oxidation of AR cysteine to sulfenic acid, the polyvinylidene difluoride membrane was incubated with DD (a sulfenic acid-specific reagent) tagged to biotin followed by horseradish peroxidase-tagged streptavidin. Positive identification of the spots with this technique indicated that in the ischemic hearts AR-SH is oxidized to AR-SOH (Fig. 1*B*, *ii*).

Spots corresponding to AR in lysates of hearts subjected to 30 min of reperfusion showed a slight shift in the molecular weight and pI when compared with AR from perfused heart. On MALDI-TOF analysis, several peptides that corresponded in mass to AR fragments were identified. Significantly, the molecular mass of two of the peptides corresponded to a glutathiolated peptide (Table 1), indicating that peptides 298–308 and 178–195, both of which contain reactive cysteines, were glutathiolated. To further confirm that AR in lysates of reperfused hearts was in the glutathiolated form, the two-dimensional gel was blotted using anti-PSSG antibody. As shown in Fig. 1*B*, *iii*, the protein spot (marked AR-SSG) corresponding to AR displayed positive immunoreactivity with the antibody, indicating that a significant fraction of AR is glutathiolated in hearts during early reperfusion.

Glutathiolation of Activated AR by Glutathione S-Transferase P—Glutathiolated AR (AR-SSG) in the reperfused heart could arise from the spontaneous addition of reduced glutathione (GSH) to AR-SOH. Alternatively, glutathiolation of AR-SOH could be catalyzed by enzymes such as the GSTs. To distinguish between these possibilities, we examined the role of GSTP. GSTP is a major cardiovascular GST isoform and accounts for 25–50% of the total GST activity in mouse aorta (20) and heart (21). To assess the role of GSTP, we used GSTP-null hearts. As reported before (15), young male GSTP-null mice grow normally and have normal cardiovascular function (21). To examine the role of GSTP, hearts from GSTP-null mice were subjected to 15 min of ischemia followed by 15, 30, or 60 min of reperfusion. As shown in Fig. 1*C*, at base line with similar duration of perfusion, AR activity was slightly higher in the GSTP-null than WT hearts (Fig. 1, compare *A* and *C*). Nevertheless, the increase in AR activity was similar in WT and GSTP-null hearts. The ischemic enzyme was relatively insensitive to sorbinil, indicating AR-SOH formation. However, in contrast to WT hearts, reperfusion of ischemic GSTP-null hearts for 15 or 30 min did not result in deactivation of the enzyme (Fig. 1*C*). Both the enzyme activity and sorbinil resistance of the enzyme remained elevated. Deactivation of the enzyme to levels similar to its perfusion values was not observed even after 60 min of reperfusion. These observations indicate that deactivation of AR is delayed in GSTP-null hearts.

Proteomics analysis of AR by two-dimensional gel electrophoresis and MALDI-TOF showed that reduced AR was present in the perfused GSTP-null hearts (Fig. 1*D*, *i*). Upon 15 min of ischemia, AR-SOH forms, identified by biotin-tagged dimedone, were observed (Fig. 1*D*, *ii*). In contrast to the WT hearts (compare Fig. 1*B*), dimedone recognized two additional spots. These could correspond to the multiple additions of dimedone to the protein oxidized at multiple cysteine residues. The identity of these spots was not further confirmed. However, in contrast with WT hearts, no AR-SSG forms of the protein were observed after 30 min of reperfusion in the GSTP-null hearts (Fig. 1*D*, *iii*). Taken together, these results suggest that in comparison with WT heart glutathiolation of AR-SOH was diminished in GSTP-null mice.

To derive quantitative estimates of the distribution of AR between AR-SH, AR-SOH, and AR-SSG during ischemia and reperfusion, the extent of AR-SOH formation was calculated from percent inhibition of AR activity by sorbinil. Assuming that 1 μM sorbinil inhibits 90% of AR-SH and 20% of AR-SOH (9), we estimated that 1% of the AR in perfused WT hearts and 57% of AR in the GSTP-null hearts were in the AR-SOH form (Fig. 2*A*). In WT heart, the percentage of AR in the AR-SOH

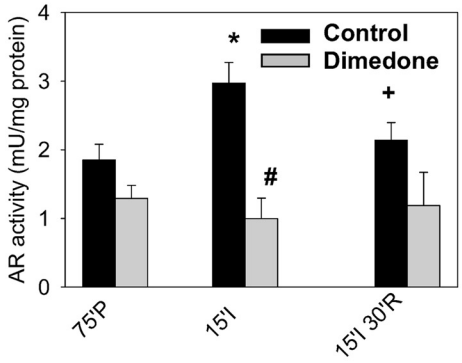
FIGURE 1. **Ischemia-induced activation of AR.** Hearts from WT (*A* and *B*) and GSTP-null (*C* and *D*) mice were excised and perfused *ex vivo* with either buffer alone for 75 min (*P*), subjected to global ischemia (*I*) for 15 min, or subjected to reperfusion (*R*) for the indicated times after 15 min of ischemia. After the indicated treatments, the hearts were snap frozen, and the AR activity was measured as described under "Experimental Procedures" in the absence or presence of 1 μM sorbinil (*A*). ', minutes. Two-dimensional Western blot analyses of AR in homogenates prepared from WT (*B*) and GSTP-null (*D*) hearts subjected either to perfusion for 75 min (*i*), 15 min of perfusion followed by 15 min of ischemia (*ii*), or 15 min of perfusion and 15 min of ischemia followed by 30 min of reperfusion (*iii*) are shown. At the end of the protocol, hearts were homogenized, and proteins were resolved by two-dimensional gel electrophoresis. Western blots (*WB*) were developed using anti-AR antibody, biotin-labeled DD and horseradish peroxidase-tagged streptavidin, or anti-PSSG antibody as indicated. *Arrows* indicate reduced AR (AR-SH), sulfenic acid-modified AR (AR-SOH), and glutathiolated AR (AR-SSG) forms of the AR protein. "?" indicates an unidentified form of AR. Values are mean ± S.D. *, $p < 0.05$ *versus* 75 min of perfusion; #, $p < 0.05$ *versus* 15 min of ischemia ($n = 3$ hearts/group).

A.

% AR activity due to the AR-SOH form

Genotype	P	I	15'R	30'R	60'R
WT	1	37	40	0	0
GSTP-null	57	81	91	100	44

B.

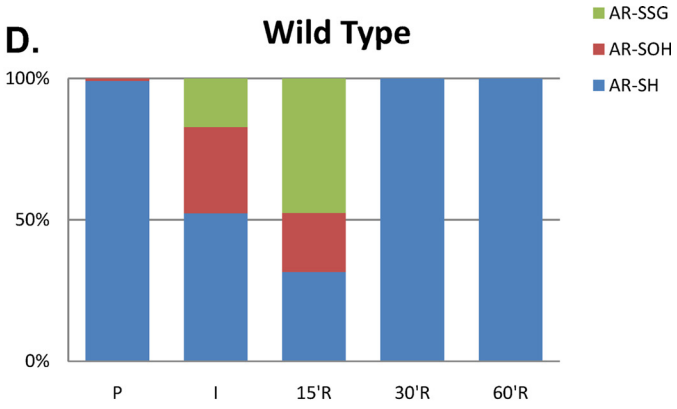


C.

Catalytic Activity

Genotype	Category	P	I	15'R	30'R	60'R
WT	Measured	**100**	**170**	**112**	**106**	**100**
WT	Predicted	100	205	214	97	97
GSTP-null	Measured	**100**	**150**	**156**	**144**	**122**
GSTP-null	Predicted	100	126	138	147	86

D.



E.

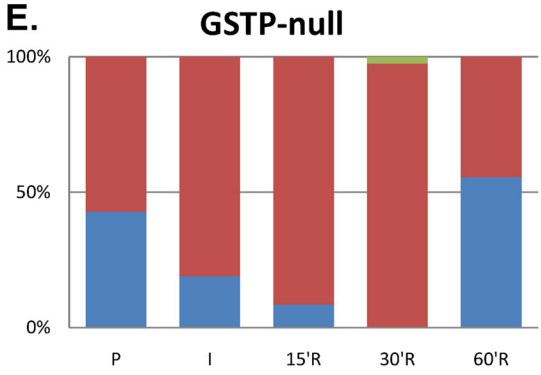


FIGURE 2. **Distribution of AR during myocardial ischemia-reperfusion.** *A*, percentage of AR activity attributable to AR-SOH formation in WT and GSTP-null hearts subjected to 75 min of perfusion (*P*), 15 min of global ischemia (*I*), or 15, 30, and 60 min of reperfusion (*R*) after 15 min of ischemia as indicated. The extent of AR-SOH formation was calculated from sorbinil inhibition data shown in Fig. 1 and was based on the assumption that 1 μM sorbinil inhibits 90% AR-SH and 20% AR-SOH activity (Equation 1). ′, minutes. *B*, inhibition of AR by dimedone. AR activity was measured in lysates of hearts subjected to 75 min of perfusion (*75′P*), 15 min of ischemia (*15′I*), or 15 min of ischemia followed by 30 min of reperfusion (*15′I 30′R*) with or without 0.5 mM dimedone. *C*, AR catalytic activity in WT and GSTP-null hearts. Measured values of AR activity are from Fig. 1 and are normalized to AR activity in perfused heart. Predicted values were calculated from the extent of AR-SOH formation (shown in *A*) and are based on the assumption that the catalytic activity of AR-SOH is 4-fold higher than that of AR-SH. The mismatch between the predicted and the measured values (highlighted in *yellow*) was used to calculate the percentage of AR in the inactive AR-SSG form. The calculated distribution of different AR forms during ischemia-reperfusion in WT (*D*) and GSTP-null (*E*) hearts is shown. Values in *B* are mean ± S.D. *, $p < 0.05$ *versus* perfused hearts; #, $p < 0.05$ *versus* ischemic hearts without dimedone treatment; +, $p < 0.05$ *versus* ischemic hearts ($n = 3$).

form increased to ~37% during ischemia and to ~40% after 15 min of reperfusion. Because the sorbinil sensitivity of the enzyme was restored after ≥30 min of reperfusion, we estimate that all the AR activity in these hearts was due to AR-SH. In contrast, nearly 80% of the enzyme was sorbinil-insensitive in ischemic GSTP-null hearts, and this value increased to 100% after 30 min of reperfusion, indicating that despite reperfusion most of the AR remains in the AR-SOH form in GSTP-null hearts.

Changes in AR activity expected from the distribution of AR between AR-SH, AR-SOH, and AR-SSG were calculated as described under "Experimental Procedures." In this procedure, first the extent of AR activation (AR-SOH) was estimated from the percent inhibition by sorbinil as described above. Next, to validate this procedure, inhibition of AR activity in the heart by dimedone was studied. Dimedone binds and inhibits AR-SOH but not AR-SH (9). Thus, the extent of inhibition by dimedone is an additional index of AR-SOH formation. Treatment with dimedone led to a small, but statistically insignificant, decrease in AR activity in the perfused heart (Fig. 2*B*). This extent of inhibition (<20%) is consistent with the nonspecific effect of dimedone on the activity of the pure AR-SH (see Ref. 9 and Fig. 3*C*). In ischemic heart lysates, the extent of inhibition of AR activity was 67%, indicating that a significant fraction of enzyme in the ischemic heart (30–50%) was in the dimedone-reactive, AR-SOH form. In contrast, most of the AR activity in reperfused heart was insensitive to dimedone. Thus, estimates of AR-SOH formation provided by sorbinil sensitivity were similar to those obtained by dimedone inactivation. Nonetheless, because of the larger error in dimedone-induced inactivation and the nonspecific effects of dimedone, calculations of the extent of enzyme activation were based on measurements of sorbinil sensitivity and not dimedone inhibition.

As shown in Fig. 2*C*, the predicted enzyme activity values based on sorbinil sensitivity were in agreement with the measured values except in WT hearts subjected to 15 min of ischemia or 15 min of reperfusion. In these hearts, the measured activity was lower than the calculated activity. We suggest that this could be attributed to loss of AR activity caused by glutathiolation in the WT hearts during ischemia and early reperfusion (Fig. 1*B*, *iii*). This view is supported by the observation that in GSTP-null hearts in which little or no AR-SSG was observed (Fig. 1*D*, *iii*) there was good agreement between the measured and the predicted activities. Estimates of the relative abundance of the various forms of AR during ischemia-reperfusion calcu-

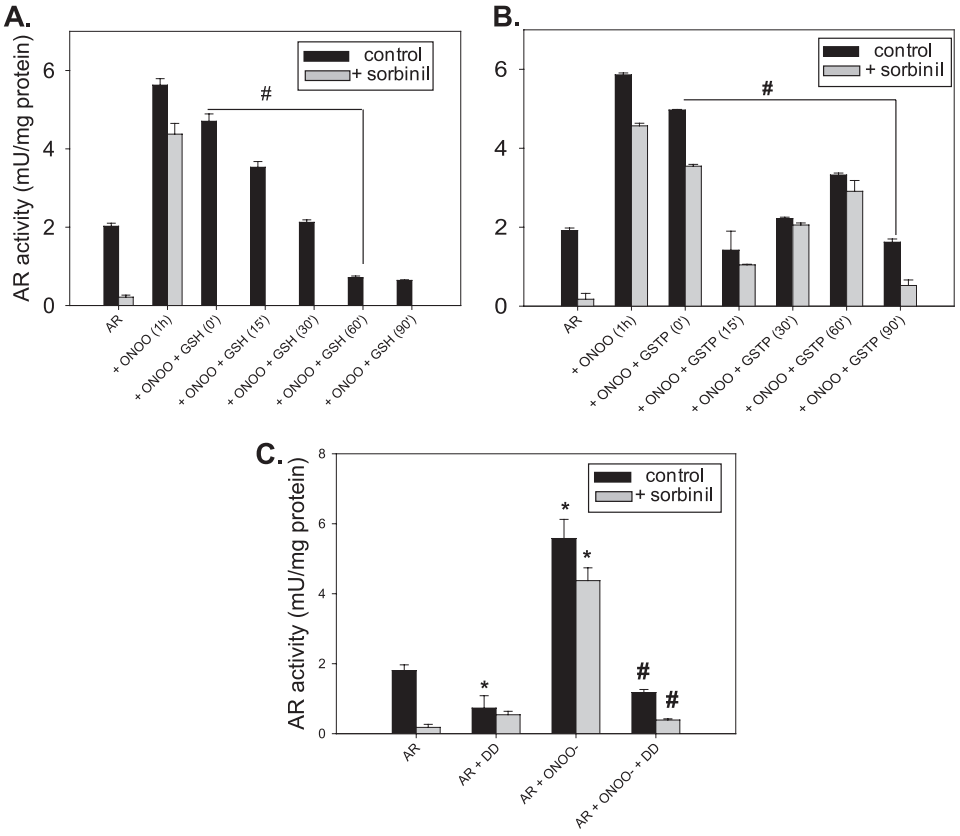


FIGURE 3. **Role of GSTP in glutathiolation of activated AR.** Human recombinant AR was treated with 0.1 mM peroxynitrite for 1 h as described under "Experimental Procedures." The peroxynitrite-modified enzyme was then incubated with 2 mM GSH (*A*) or GSH and human recombinant GSTP (*B*) in 50 mM phosphate buffer, pH 7.0. Aliquots were withdrawn from the reaction mixture at the indicated time for measurement of AR activity with 10 mM DL-glyceraldehyde as substrate without and with sorbinil (1 μM). ′, minutes. *C*, effect of DD on reduced and oxidized AR. Reduced or peroxynitrite-treated AR was incubated with 0.5 mM DD; after 60 min of incubation, aliquots were withdrawn from the reaction mixture; and AR activity was measured without and with sorbinil (1 μM). Incubation with GSTP or GSH alone did not affect AR activity. Values are mean ± S.D. *, $p < 0.05$ *versus* untreated AR; #, $p < 0.05$ *versus* $ONOO^-$-treated AR ($n = 3$).

lated using Equations 2–4 are shown in Fig. 2, *D* and *E*, for WT and GSTP-null hearts.

To examine the role of GSTP in catalyzing the glutathiolation of activated AR, recombinant, His tag-purified human AR was oxidized by $ONOO^-$. Our previous studies show that incubation of AR with $ONOO^-$ results in the oxidation of AR to AR-SOH (13). In agreement with these observations, we found that incubation of AR with $ONOO^-$ for 1 h led to partial activation of AR, resulting in a 3-fold increase in AR activity (Fig. 3*A*). In comparison with the reduced enzyme, $ONOO^-$-treated AR was relatively insensitive to sorbinil, indicating enzyme activation (Fig. 3*A*). Upon incubation with GSH alone, a progressive decrease in the enzyme activity was observed. After 90 min, 85–90% of AR activity was inhibited (Fig. 3*A*). Incubation with dimedone inhibited the activity of the $ONOO^-$-modified AR, confirming AR-SOH formation (Fig. 3*C*). When the $ONOO^-$-treated AR was incubated with GSH and GSTP, the decrease in enzyme activity was faster than in the enzyme that was incubated with GSH alone. More than 70% of the enzyme activity was lost after only 15 min of incubation (Fig. 3*B*). Thereafter, a slight increase in enzyme activity was transiently observed, but most of the enzyme remained inactive after 30 min of incubation. Treatment with GSH restored the sorbinil sensitivity, indicating that the enzyme was converted back to the AR-SH form. These observations suggest that GSTP accelerates the rate of deactivation of activated AR. Because the enzyme regained sorbinil sensitivity, we conclude that the enzyme was deactivated rather than simply inhibited.

To examine whether changes in enzyme activity were due to changes in the glutathiolation status of the protein, recombinant AR was incubated with $ONOO^-$ and then treated with GSH in the absence and presence of GSTP. Glutathiolation of the enzyme was determined by Western blot analysis using anti-PSSG antibody. As shown in Fig. 4*A*, when the $ONOO^-$-treated enzyme was incubated with GSH in the absence of GSTP, AR-SSG was detected within 30 min after which the protein remained in this form for the next 60 min. When incubated with GSH and GSTP, maximal glutathiolation was observed within 15 min, and the level of AR-SSG was slightly decreased after 30, 60, and 90 min (Fig. 4*B*). These observations suggest that glutathiolation of AR was accelerated by GSTP and that in the presence of GSTP AR-SSG has a greater tendency to spontaneously undergo dethiolation than the enzyme glutathiolated by GSH alone. The spontaneous dethiolation of AR-SSG in the presence of GSTP is consistent with the activity data (Fig. 3*B*) showing spontaneous regeneration of the deactivated enzyme. These observations suggest that GSTP-catalyzed glutathiolation is more specific and, therefore, more readily reversible than the uncatalyzed reaction.

To identify the molecular forms of AR generated during activation and deactivation, we used electrospray mass spectrometry. The ESI^+/MS spectra of reduced AR showed well resolved species with a molecular mass of 37,883 Da, corresponding to His-tagged AR-SH (Fig. 5*A*, *i*). A minor form of AR with a mass of 37,924 Da was also observed, but the identity of this form remains unclear. Incubation of AR with $ONOO^-$ for 1 h resulted in a +16-Da increase in the molecular mass, consistent with the formation of a single sulfenic acid form of the protein (Fig. 5*A*, *ii*). Addition of GSH to this species resulted in the generation of an additional +306-Da species, indicating glutathiolation of AR-SOH to AR-SOSG (Fig. 5*A*, *iii*). A significant fraction of the protein, however, remained as AR-SOH. However, when the same experiment was repeated in the presence of GSTP, AR-SOH was readily converted to AR-SSG within 15 min (Fig. 5*B*, *i–iii*). No AR-SOH or AR-SOSG forms were observed after 15 min, but a small fraction of the enzyme

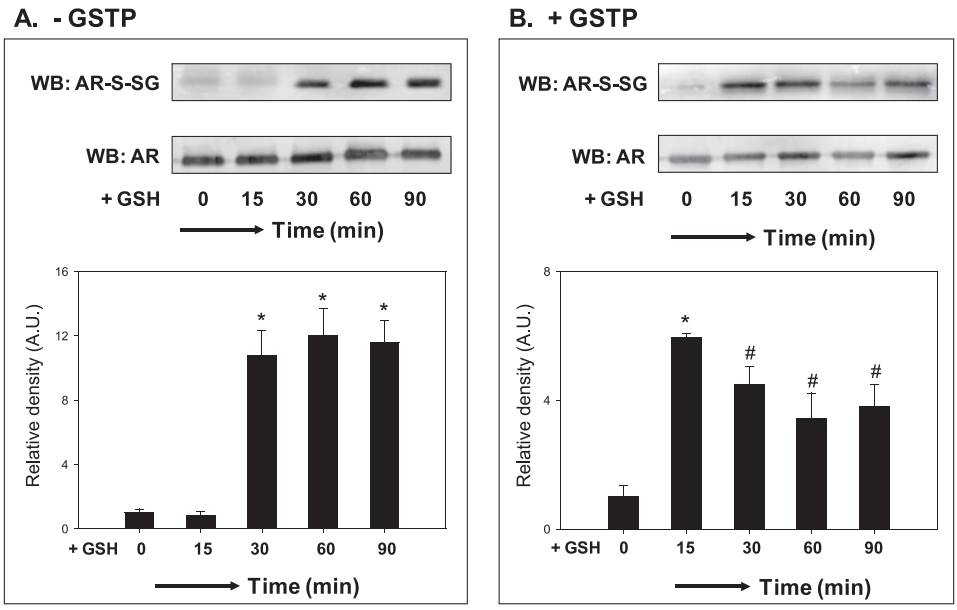


FIGURE 4. **GSTP-dependent AR glutathiolation.** Recombinant human AR was reduced with 0.1 M DTT and desalted by passing through a PD-10 column. The sulfhydryl group of AR was converted to sulfenic acid by treatment with peroxynitrite. The oxidized enzyme was then incubated with GSH (*A*) or GSH + GSTP (*B*). Aliquots of the reaction mixture were withdrawn at the indicated times, and the extent of glutathiolation was measured. The *upper panels* show Western blots (*WB*) of the aliquots developed using anti-AR and anti-PSSG antibodies, and measurements of band intensity are shown in the *lower panels*. Values are mean ± S.D. *, $p < 0.05$ *versus* time 0; #, $p < 0.05$ *versus* 15 min ($n = 3$ experiments). *A.U.*, arbitrary units.

appeared in the reduced form. Continuing the incubation for 90 min did not change the distribution of the enzyme within the two forms (data not shown). Collectively, these data indicate that GSTP accelerates glutathiolation of AR-SOH.

Role of Glutaredoxin in Deglutathiolation of AR—Previous studies show that GRX can catalyze deglutathiolation of proteins (22). To examine whether GRX can catalyze the reduction of AR-SSG to AR-SH, we first tested whether AR-SSG formed *in vitro* could be reduced by GRX. To generate AR-SSG, the enzyme was first oxidized to sulfenic acid by $ONOO^-$ and then incubated with GSH. Incubation of AR-SH with $ONOO^-$ resulted in a 2–3-fold increase in enzyme activity and a decrease in sorbinil sensitivity (Fig. 6*A*), indicating oxidation and activation of the enzyme. Addition of GSH to the $ONOO^-$-treated enzyme resulted in the loss of enzyme activity, indicating that the protein may be glutathiolated. Upon incubation with GSH, NADPH, and glutathione reductase, there was further loss of enzyme activity due to continued glutathiolation. However, a time-dependent partial recovery of enzyme activity and sorbinil sensitivity was observed, indicating that the enzyme was spontaneously deglutathiolated. When the same experiment was repeated in the presence of GRX, the loss of activity was less, and the enzyme activity was regained to near control levels within 60 min with partial recovery of sorbinil sensitivity (Fig. 6*B*). These data indicate that GRX accelerates the conversion of AR-SSG to AR-SH.

To examine whether changes in AR activity were due to changes in the glutathiolation status of the protein, recombinant AR was incubated with $ONOO^-$ and then treated with GSH, glutathione reductase, and NADPH in the absence and presence of GRX. Glutathiolation of the enzyme was determined by Western blot analysis using the anti-PSSG antibody. As shown in Fig. 7, when $ONOO^-$-treated AR was incubated with GSH, a decrease in AR-SSG was first detected at 30 min after which the protein continued to be deglutathiolated over the next 60 min. When incubated with GSH in the presence of GRX, significant deglutathiolation was observed within 15 min, and the level of AR-SSG was continuously decreased at 30, 60, and 90 min (Fig. 7*B*). These observations suggest that GRX accelerates deglutathiolation of AR-SSG.

The molecular species generated during GRX catalysis were examined by ESI^+/MS. As shown in Fig. 7*C*, *i*, AR-SH incubated with $ONOO^-$ followed by GSH and GSTP appeared mostly as AR-SSG as indicated by a +307-Da increase in mass (38,190 Da) compared with the native reduced enzyme (37,883 Da). Incubation with GSH, glutathione reductase, and NADPH did not result in significant change in the intensity of the 38,190-Da ion (Fig. 7*C*, *ii* and *iii*). In comparison, incubation of AR-SSG with the same reaction mixture containing GRX resulted in the reappearance of the native reduced AR ion, which was similar in intensity to AR-SSG at 15 min (Fig. 7*D*, *i–iii*). After 90 min, the major ion in the mixture corresponded to AR-SH with only trace levels of AR-SSG (Fig. 7*D*, *vi*). These observations suggest that GRX catalyzes the reduction of AR-SSG to AR-SH.

To examine the *in vivo* role of GRX in regulating AR thiolation, activation of AR was examined in GRX-TG and GRX-null mice. GRX-TG hearts had significantly lower AR activity after 60 min of perfusion compared with AR activity in WT heart. When subjected to 15 min of ischemia, AR was activated in the WT hearts but not in GRX-TG hearts (Fig. 8*A*). Indeed, in comparison with WT hearts, the AR activity remained significantly (~2-fold) lower. In ischemic GRX-TG hearts, the AR activity was similar to that in the hearts subjected to perfusion alone. Upon reperfusion, AR was deactivated in WT hearts; however, in GRX-TG hearts, reperfusion led to a slight increase in AR activity (Fig. 8*A*). These observations indicate that an increase in GRX prevents AR activation in the ischemic heart, but it promotes AR activation in the reperfused heart.

In GRX-null hearts, the level of AR activity was similar to that in WT hearts (Fig. 8*B*). Moreover, when these hearts were subjected to 15 min of ischemia, the extent of AR activation was very similar to that observed in WT hearts. However, upon reperfusion, the loss of AR activation was slower in the GRX-null than in WT hearts such that after 30 min of reperfusion the activity of AR was significantly higher in GRX-null hearts than in WT hearts (Fig. 8*B*). These results are consistent with the idea that lack of GRX maintains AR in the activated state and prevents deactivation of the enzyme during reperfusion.

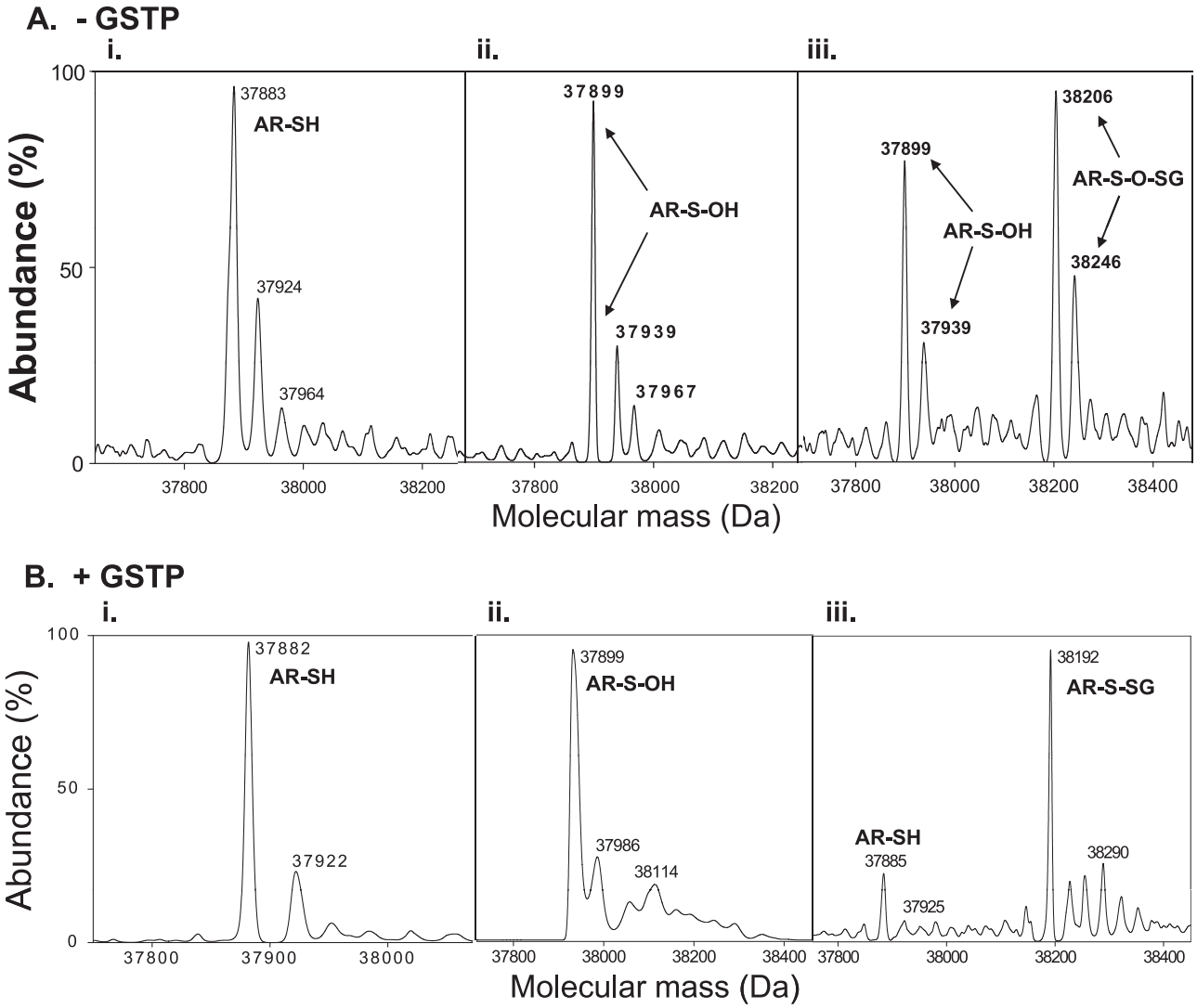


FIGURE 5. **Glutathiolation of AR protein by GSTP.** Reduced recombinant AR treated with $ONOO^-$ was desalted and treated with GSH (*A*) or GSH + GSTP (*B*). Aliquots were withdrawn at the indicated times and analyzed by ESI^+/MS. *A*, deconvoluted spectra of unmodified, reduced AR protein (37,883 Da) and a minor unidentified peak (37,924 Da) (*i*), AR incubated with $ONOO^-$ showing a 16-Da increase in mass (AR-SOH, 37,899 Da) (*ii*), and AR after incubation with 2 mM GSH for 15 min showing the AR-SOH (37,899 Da) and the AR-SOSG (38,206 Da) forms of the protein (*iii*). *B*, AR glutathiolation in the presence of GSTP showing unmodified AR (37,882 Da) (*i*), reduced AR incubated with 0.1 mM $ONOO^-$ showing AR-SOH (37,899 Da) (*ii*), and AR-SOH incubated for 15 min with 2 mM GSH and GSTP showing both AR-SH (37,885 Da) and the AR-SSG (38,192 Da) forms (*iii*).

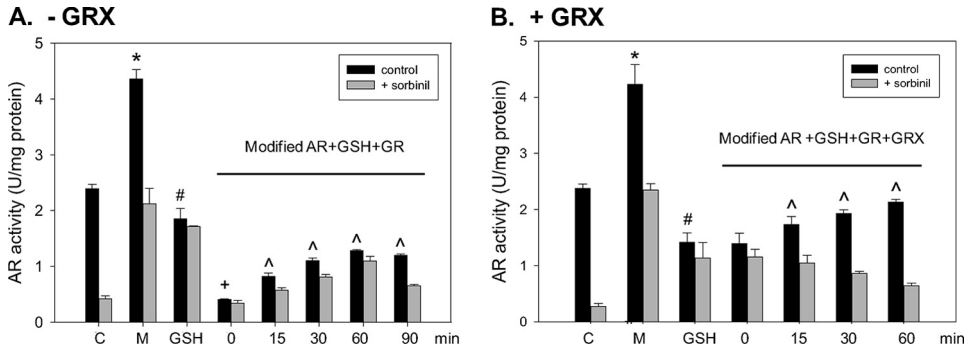


FIGURE 6. **Reduction of AR-SSG by GRX.** Reduced AR was incubated with 0.1 mM $ONOO^-$. After incubation, the mixture was desalted to remove $ONOO^-$. Catalytic activity of the reduced (*C*) and modified (*M*) enzyme with or without 1 μM sorbinil is shown. The modified enzyme was then incubated with 2 mM GSH alone for 15 min; after that, glutathione reductase (*GR*) and NADPH were added to GSH-containing modified enzyme, and the mixture was then incubated at 25 °C in the absence (*A*) or presence (*B*) of GRX as indicated. Aliquots from the reaction mixture were withdrawn at the indicated time intervals and assayed for AR activity with and without sorbinil (1 μM). Data are expressed as mean ± S.D. *, $p < 0.05$ *versus* AR (*C*) alone; #, $p < 0.05$ *versus* AR + $ONOO^-$ (*M*); +, $p < 0.05$ *versus* AR + $ONOO^-$ + GSH (*GSH*); ▲, $p < 0.05$ *versus* modified AR + glutathione reductase + GSH (0 min) or modified AR + glutathione reductase + GRX + GSH (0 min); $n = 3$ experiments at each time point.

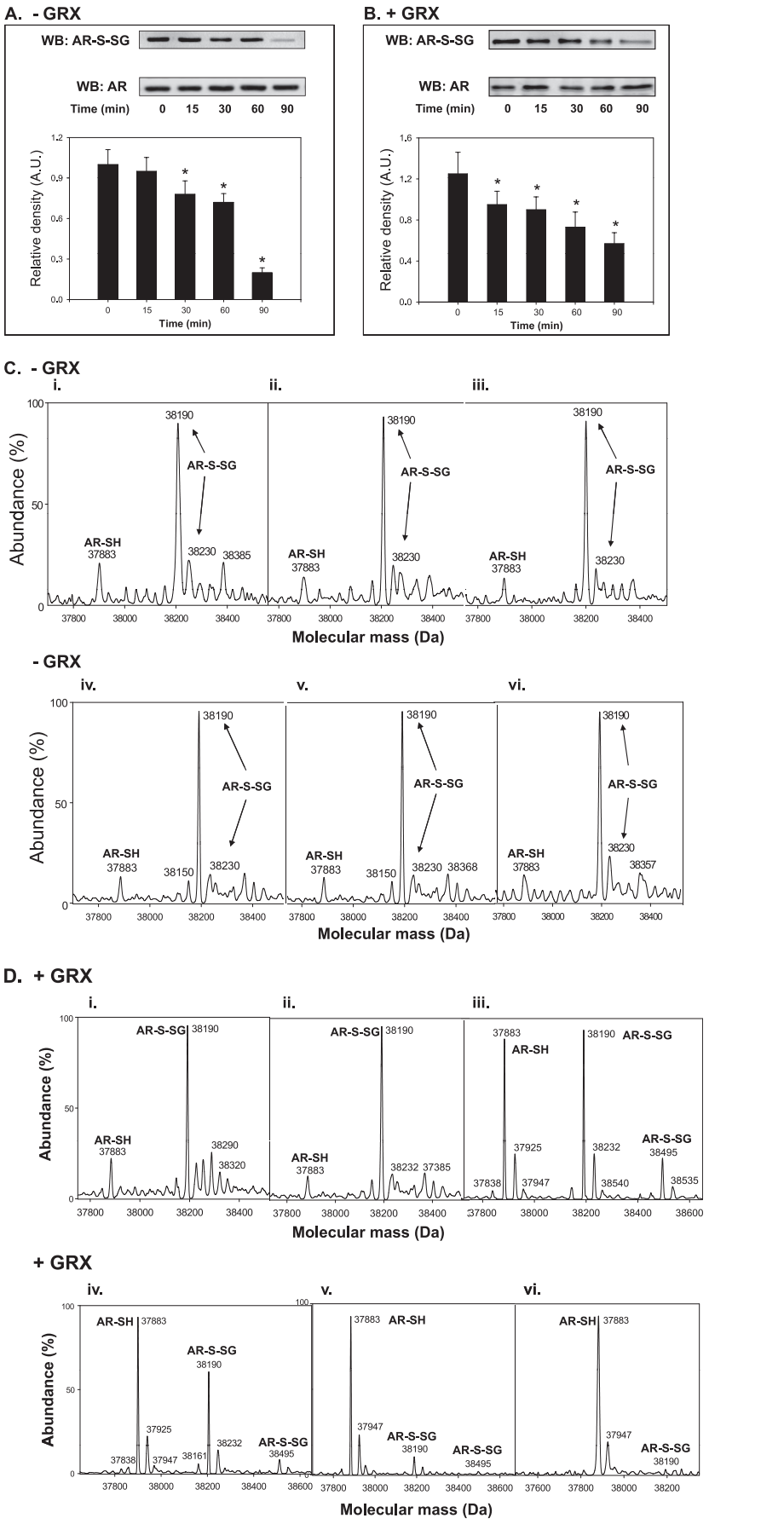
Association of AR with GSTP and GRX—Our investigation so far showed that deactivation of AR in the ischemic heart is catalyzed sequentially by GSTP and GRX. If this is the case, we expect that AR may be transiently associated with these enzymes during ischemia and/or reperfusion. To examine the association of AR with these proteins, we used AR-TG mice. In these mice, the expression of the AR transgene is driven by the α-myosin heavy chain promoter so that the protein is expressed only in cardiac myocytes. Moreover, to facilitate immunoprecipitation, a His tag leader sequence was included in the

vector. Despite 2– 4-fold higher levels of AR protein in the AR-TG hearts, the cardiac function in these mice is normal, and they display no obvious phenotype (23). When subjected to 15 min of global ischemia, a 2-fold increase in enzyme activity was observed (Fig. 9*A*). The relative increase in AR activity was similar to that in the WT hearts, but the overall magnitude of the enzyme activity was greater due to an increased AR protein level (Fig. 9*A*). Upon reperfusion, AR was deactivated to an extent similar to that observed in WT hearts. To examine the extent of glutathiolation and association with GSTP and GRX, AR was immunoprecipitated with an anti-His tag antibody and separated by SDS-PAGE, and Western blots were developed using anti-PSSG, anti-GSTP, or anti-GRX antibody. Western blots with anti-AR antibody were used to normalize levels of the immunoprecipitated proteins to control for variations in the efficiency of immunoprecipitation.

During perfusion, only basal levels of immunoreactivity with anti-PSSG were observed; however, higher levels of PSSG reactivity were detected in lysates from ischemic hearts that returned to base-line values in the reperfused heart extracts (Fig. 9*B*). Similarly, the levels of GSTP in the immunoprecipitate were increased in ischemic hearts but returned to preischemic values in the reperfused hearts (Fig. 9*C*, *ii*). The extent of reactivity of the anti-GRX antibody in immunoprecipitates of the perfused and ischemic heart lysates was similar; however, greater levels of GRX were immunoprecipitated with AR in the reperfused hearts. Group data are shown in Fig. 9*C*, *i–iii*. These observations support the notion that the association between AR and GSTP is increased during ischemia, whereas more GRX is associated with AR during reperfusion than before or during ischemia.

DISCUSSION

Results of this study show that AR is activated in the ischemic heart,



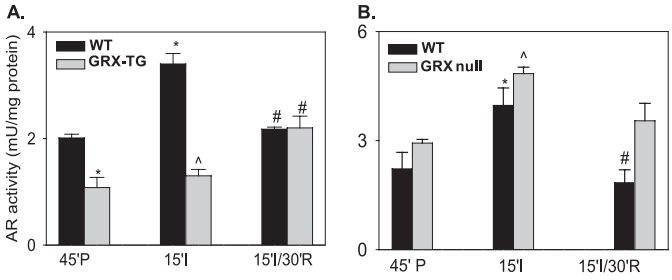


FIGURE 8. **Regulation of reperfusion-induced AR deactivation by GRX in heart.** Isolated perfused hearts from WT and GRX-TG (*A*) and WT and GRX-null (*B*) mice were subjected to 15 min of global ischemia alone (*15′I*) or 15 min of ischemia followed by 30 min of reperfusion (*15′I/30′R*). *45′P*, 45 min of perfusion. WT hearts subjected to the same treatment were used as controls. AR activity was measured as described under "Experimental Procedures." Values are mean ± S.D. ($n = 3$ experiments). *, $p < 0.05$ compared with perfusion only; ▲, $p < 0.05$ compared with 15 min of ischemia in WT; #, $p < 0.05$ compared with 15 min of ischemia.

and then it is deactivated during early reperfusion. This process was found to be regulated by GSTP and GRX. These enzymes accelerated AR deactivation *in vitro*, and changes in their expression in the heart disrupted the activation-deactivation cycle of AR during myocardial ischemia-reperfusion. Based on this evidence, we propose a general model for AR deactivation (Scheme 1). In this model, reduced AR (AR-SH) is activated by oxidation to AR-SOH during ischemia. However, during reperfusion, AR-SOH is reduced back to AR-SH. During this reduction cycle, AR-SSG is formed as an intermediate generated by GSTP-assisted catalysis. Glutathione bound to the enzyme is then removed by GRX, resulting in the appearance of the reduced, unactivated enzyme.

Protein sulfenic acids are usually generated during excessive protein oxidation *in vitro* (24) or under conditions of high oxidative stress, *e.g.* tissues exposed to supraphysiological concentrations of peroxides (25). They are also formed as transient intermediates during the catalytic cycle of enzymes such as peroxiredoxins (26) or methionine sulfoxide reductases (Msr) (27) or transcription factors such as the bacterial OhrR (28). Nevertheless, the metabolic fate of protein sulfenic acids is poorly understood. Sulfenic acids generated at the active sites of peroxiredoxins are reversible (29) and are converted into intramolecular disulfides, which are then reduced by thioredoxins (30). Sulfenic acids generated in yeast 2-Cys Prx undergo ATP-dependent reduction (31), whereas sulfenic acid intermediates of Msr are directly reduced by thioredoxin (32). In the cases of 5′-methylthioadenosine phosphorylase (33) and protein-tyrosine phosphatase 1B (34, 35), the sulfenic acids are stabilized to sulfenyl amide intermediates. Sulfenic acids generated in mitogen-activated protein kinase phosphatase (MKP3) (36) and Cdc25 phosphatase (37) are trapped as disulfides. However, the fate of other protein sulfenic acids generated at non-catalytic sites remains unknown. In addition, it is unclear whether the proteins modified by sulfenic acid formation are reduced back to their normal reduced form or whether they are degraded by proteolysis as is the case for other forms of protein oxidations. In the case of AR, we found that sulfenic acids generated in the enzyme during ischemia were removed upon reperfusion. Because the enzyme activity returned to its preischemic level without any changes in AR protein (9), it appears that AR-SOH is not degraded and that sulfenic acids generated in AR during ischemia are enzymatically removed upon reperfusion.

Previous studies show that protein sulfenic acids have a high propensity to condense with proximal thiol groups to form intra- or intermolecular disulfide bonds (38). In AR, Cys-298 is the main residue involved in sulfenic acid formation. This residue is close to Cys-303; however, there was no evidence for the formation of a disulfide bond between the two neighboring cysteines. Conversion of the sulfenic form to an intramolecular disulfide form may not be an efficient mechanism for the regeneration of AR because during ischemia both these cysteines could undergo sulfenic acid formation. Therefore, assisted reduction may be required. Indeed, our studies showing that AR-SOH undergoes glutathiolation both *in vitro* and *in vivo* suggest that there is no disulfide bond formation and that adduction with GSH is required for reducing AR-SOH back to AR-SH.

Reduced glutathione adds readily to protein sulfenic acids (39), and it has been suggested that glutathiolated proteins generated during oxidative stress are formed via sulfenic acid intermediates (38). Nevertheless, glutathiolation of protein sulfenic acids has not been directly demonstrated. Thus, our data showing complete conversion of AR-SOH to AR-SSG in the presence of GSH (Fig. 5) represent the first direct demonstration of protein sulfenic acid glutathiolation. The *in vivo* relevance of this reaction is provided by data showing that AR-SOH, which is generated during ischemia, is converted to AR-SSG during reperfusion. These observations suggest that glutathiolation may be a significant fate of protein sulfenic acids generated *in vivo*. Nevertheless, at least for AR, it appears that unassisted glutathiolation is relatively inefficient. *In vitro*, 90 min were required for complete glutathiolation of AR-SOH by GSH alone, but in the presence of GSTP, glutathiolation was essentially complete within 15 min (Fig. 5). Moreover, although the addition of GSH to AR-SOH resulted in the appearance of AR-SOSG (Fig. 5*A*), no such forms were observed in the presence of GSTP (Fig. 5*B*), indicating that enzymatic glutathiolation by GSTP prevents the formation of spurious intermediates and promotes monogenic conversion of AR-SOH to AR-SSG. *In vivo* evidence also supports the idea that GSTP accelerates gluta-

FIGURE 7. **Deglutathiolation of AR-SSG by GRX.** AR reduced with 0.1 M DTT and incubated with 0.1 mM $ONOO^-$ was desalted and then incubated with 2 mM GSH, glutathione reductase (*GR*), and NADPH at 25 °C in the absence (*A*) or presence (*B*) of GRX. Aliquots from the reaction mixture were withdrawn at the indicated times, and glutathiolated AR was assayed by Western blot (*WB*) using anti-PSSG antibody. Values are mean ± S.D. *, $p < 0.05$ *versus* 0-min time point ($n = 3$ experiments at each time point). Aliquots of the reaction mixture withdrawn at regular time intervals were also analyzed by ESI/MS. *C* (*i–vi*) shows ESI/MS spectra of AR incubated with $ONOO^-$, GSH, and GSTP showing both the native (37,883 Da; minor peak) and the AR-SSG form (38,190 Da; major peak) (*i*). Incubation of the AR-SSG form with glutathione reductase for 0 **(*ii*)**, 15 **(*iii*)**, 30 **(*iv*)**, 60 **(*v*)**, and 90 **(*vi*)** min shows the major form AR-SSG (38,190 Da) and a minor form corresponding to native AR (37,883 Da). Incubation of AR-SSG, generated by incubating AR with $ONOO^-$ **(*D*, *i*)**, with GSH, GSTP, and GRX **(*D*, *ii*)** for 0 min shows both the AR-SSG (38,190 Da; major peak) and the native forms (37,883 Da; minor peak), whereas incubation for 15 min (*D*, *iii*) shows two equal peaks of the native (37,883 Da) and AR-SSG (38,190 Da) forms of the protein. Incubation of AR-SSG with 2 mM GSH and GRX for 30 (*D*, *iv*), 60 (*D*, *v*), and 90 min (*D*, *vi*) shows the major reduced AR-SH form (37,883 Da) and the minor AR-SSG form (38,190 Da) of the protein. *A.U.*, arbitrary units.

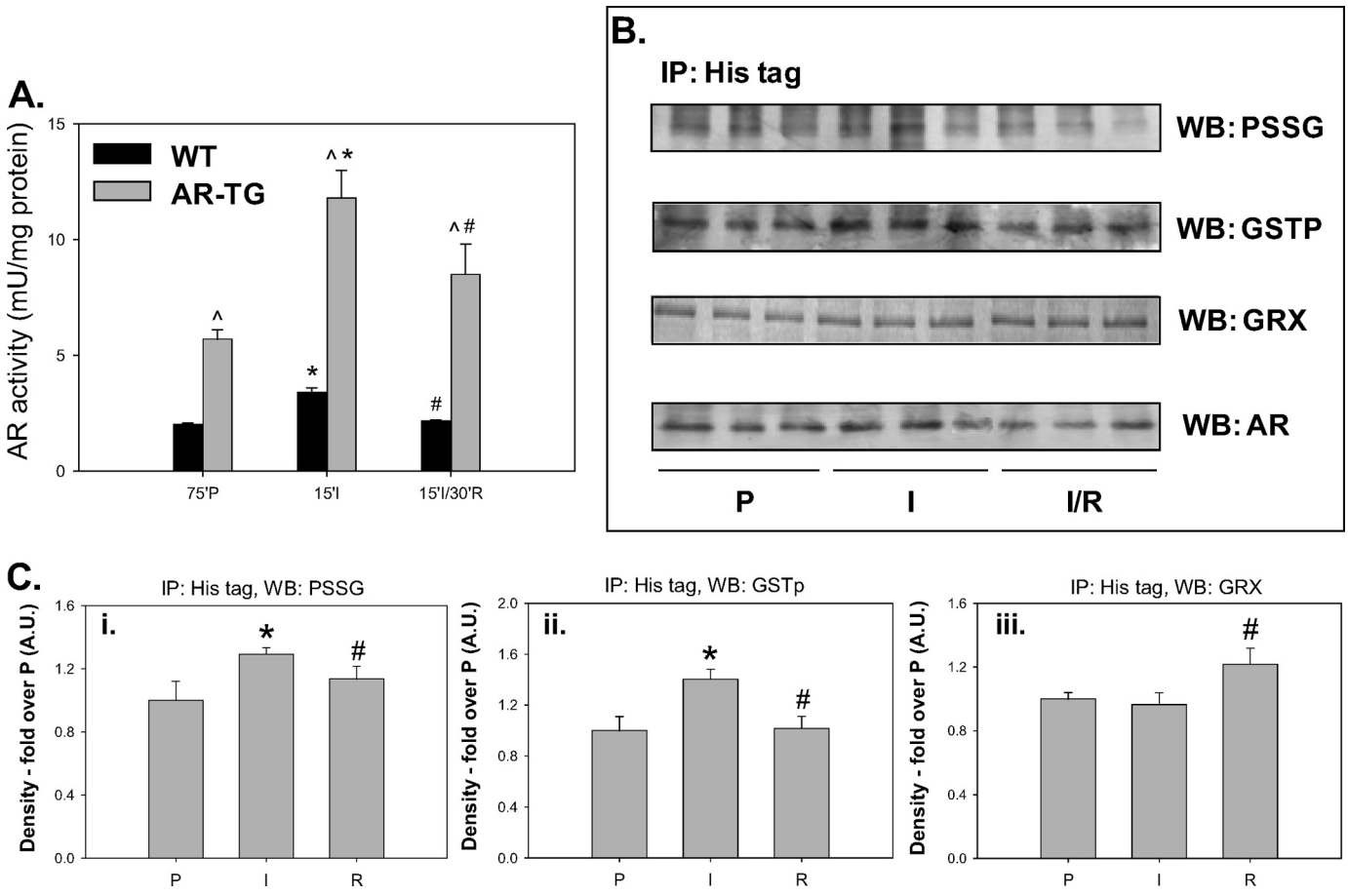


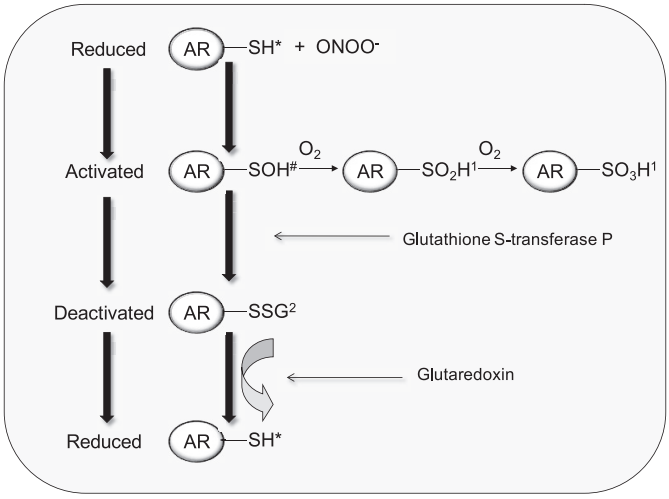
FIGURE 9. **Association of AR with GSTP and GRX during myocardial ischemia-reperfusion.** *A*, AR activity was measured in WT and AR-TG heart homogenates that were subjected to 15 min of ischemia alone (*15′I*) or 15 min of ischemia followed by 30 min of reperfusion (*15′I/30′R*). The 75 min of perfusion (*75′P*) group data served as controls. *B*, the extent of AR glutathiolation and interaction with GRX and GSTP was assessed by immunoprecipitating AR from perfused (*P*), ischemic (*I*), or ischemic-reperfused hearts (*I/R*). Western blots (*WB*) of the immunoprecipitate (*IP*) were developed using anti-PSSG, anti-GSTP, and anti-GRX antibodies. *C*, levels of each of these proteins (*i–iii*) were normalized to the amount of AR detected in the immunoprecipitate measured by Western blotting using anti-His antibodies that recognize the AR transgene in the heart. No AR-His protein, GRX, or GSTP was immunoprecipitated with an unrelated antibody. Values are mean ± S.D. ($n = 3$ experiments). *, $p < 0.05$ *versus* 75 min of perfusion; ▲, $p < 0.05$ *versus* 75 min of perfusion; #, $p < 0.05$ *versus* 15 min of ischemia. *A.U.*, arbitrary units.

thiolation of AR-SOH. In GSTP-null mice, deactivation of activated AR (AR-SOH) was delayed (Fig. 1, *A* and *C*), and the extent of AR-SSG formation was decreased (Fig. 1, *B* and *D*) in reperfused hearts.

GSTP is a member of a large family of GSTs that catalyzes the addition of GSH to electrophilic xenobiotics (40). It has high activity with low molecular weight aldehydes such as acrolein and base propenals (41). In addition, GSTP binds to and regulates c-Jun N-terminal kinase (JNK) and peroxiredoxin VI via protein-protein interactions (42, 43). Recent studies show that GSTP regulates the protein *S*-glutathiolation in cells under oxidative and nitrosative stress (44). Our findings support this role of GSTP and directly demonstrate for the first time GSTP-catalyzed transfer of GSH to a protein sulfenic acid (Fig. 5*B*). Further studies are required to determine whether GSTP catalyzes the glutathiolation of sulfenic acids generated in proteins other than AR.

In addition to glutathiolation, GSTP can also catalyze protein deglutathiolation. However, we found that incubation of AR-SOH with GSH with or without GSTP led to the appearance of trace quantities of AR-SH, but a large fraction of the protein remained in the glutathiolated state (AR-SSG). Hence, at least in the case of AR, GSTP does not appear to be an efficient deglutathiolase. Moreover, AR-SSG did not seem to spontaneously revert back to AR-SH by intramolecular reduction. Instead, our data suggest that deglutathiolation of AR is facilitated by GRX. We found that the GRX accelerates deactivation (recovery of sorbinil sensitivity; Fig. 6) of AR as well as reduction of AR-SSG to AR-SH (Fig. 7).

Glutaredoxins are general thiol-disulfide oxidoreductases. These enzymes catalyze the reduction of protein disulfides and mixed disulfides. They also catalyze dethiolation of protein-SSG (22). However, only a few proteins have been shown to be deglutathiolated by GRX under physiological contexts. For instance, *S*-thiolation of IκB kinase-β has been shown to be reversed by GRX (45). Our result showing that AR-SSG is readily dethiolated in the presence of GRX provides yet another example of the deglutathiolase activity of GRX. The *in vivo* role of GRX in regulating AR dethiolation is supported by our observations showing that ischemia-induced AR activation was prevented in GRX-TG hearts and the reperfusion-induced AR deactivation was delayed in GRX-null hearts. These observations suggest that changes in GRX expression dysregulate the activation-deactivation cycle of AR during myocardial ischemia-reperfusion.



SCHEME 1. **Mechanism for glutathiolation and deglutathiolation of AR.** Reduced AR (AR-SH) is oxidized by peroxynitrite ($ONOO^-$ generated during ischemia) to AR-SOH (activated form). The sulfenic acid form of AR (AR-SOH) could be irreversibly oxidized to sulfinic acid (AR-SO_2H) and sulfonic acid (AR-SO_3H) forms. The reversible, sulfenic acid form of AR is deactivated to an inactive form by glutathiolation (AR-SSG) of the sulfenic acid residue via a reaction catalyzed by GSTP in the presence of GSH. The AR-SSG is recycled to the native, reduced form (AR-SH) by GRX in the presence of GSH. *, sorbinil-sensitive AR with basal activity; #, dimedone-sensitive activated AR; *1*, catalytic activity unknown; *2*, enzymatically inactive AR.

Our findings suggest that AR deactivation requires both GSTP and GRX and that these two enzymes work in tandem during specific phases of ischemia and reperfusion (Scheme 1). This scheme is supported by immunoprecipitation experiments showing that the association between AR and GSTP is increased during ischemia, whereas the binding of GRX to AR was increased during reperfusion (Fig. 8). These data suggest that activated AR undergoes two sequential reactions in a temporally segregated manner. The first reaction involves the addition of GSH to AR-SOH by GSTP. This reaction is followed by GRX-catalyzed removal of glutathione from AR-SSG. Both *in vitro* and *in vivo* data are consistent with this scheme. The observation that AR deactivation was delayed in GSTP-null heart suggests that GSTP accelerates deactivation, although it does not seem to be essential for the process. GSTP-induced deactivation, however, seems to depend upon GRX because in GRX-TG hearts no activation was observed. We suggest that this might be due to more rapid removal of AR-SSG in GRX-TG hearts such that there is no steady-state accumulation of AR-SOH. This may be due to two reasons: 1) an increase in GSTP activity due to more efficient removal of its product AR-SSG or 2) the ability of GRX to catalyze both the glutathiolation and the deglutathiolation of AR. Because GRX was unable to catalyze AR glutathiolation *in vitro*, we favor the first hypothesis. This hypothesis is also consistent with the observation that in GRX-null hearts the extent of AR activation during ischemia was increased and AR deactivation during reperfusion was delayed, indicating that a decrease in GRX-mediated deglutathiolation decreases GSTP-catalyzed glutathiolation. If this were not the case, AR-SSG formation would have increased in the GRX-null hearts, leading to a net decrease in AR activity rather than the increase we observed. Further work is required to determine how GSTP and GRX work in tandem to catalyze the deactivation of AR and whether this pathway removes other protein sulfenic acids as well.

Western blot analyses indicate that AR-SH is the major form of AR in the perfused heart, whereas during ischemia and reperfusion AR-SOH and AR-SSG are transiently generated (Fig. 1). Because different procedures and antibodies were used to identify these forms of AR, the presence of different forms of the enzyme in Western analysis could not be quantitatively related to the extent of activation or deactivation. However, our kinetic analyses provide more quantitative estimates. These show that up to 30% of the enzyme was in the AR-SOH form in the ischemic heart and that 50% of the enzyme was in the AR-SSG form during early reperfusion (Fig. 2). In contrast, in GSTP-null hearts, 80–100% of the enzyme appears to be in the AR-SOH form during ischemia and early reperfusion. These estimates indicate that the absence of GSTP inhibits AR deactivation. The accumulation of AR-SOH in the ischemic heart may represent a steady state established by oxidative processes leading to enzyme activation and the reductive processes (GSTP and GRX) promoting deactivation. Evidence for this steady state is provided by data showing that perturbations of the steady state by GRX overexpression prevent the accumulation of AR-SOH in the ischemic heart. Thus, the activation-deactivation cycle of AR seems to be driven actively by the oxidation of AR-SH to AR-SOH and regulated passively by the activities of GSTP and GRX.

The activation-deactivation cycle of AR is consistent with a protective role of this enzyme during myocardial ischemia-reperfusion. Our previous studies show that AR detoxifies aldehydes generated from oxidized lipids (10). Ischemia-reperfusion increases ROS production in the heart. These conditions favor the formation and the accumulation of lipid-derived aldehydes, which could trigger inflammation and induce tissue injury (46, 47). Hence, an increase in AR activity in the ischemic heart and during early reperfusion may be a protective mechanism for increasing the detoxification of lipid peroxidation products. Indeed, our studies show that reductive metabolism of 4-hydroxy-*trans*-2-nonenal is increased in ischemic hearts (48) and that inhibition of AR increases myocardial accumulation of lipid peroxidation products during ischemia-reperfusion (12) as well as the extent of myocardial infarction (13). Thus, activation of AR may be a mechanism to minimize ischemic injury. In contrast to this hypothesis, other investigators have reported that inhibition of AR prevents myocardial ischemia-reperfusion injury (49–51). The reasons for such directly opposite results are not clear but may relate to the insensitivity of the enzyme to pharmacological inhibition in the ischemic heart, difference in the levels of AR-regulating enzymes (GSTP, GRX, and others) in different species, and levels of oxygen leading to differences in the oxidized levels of AR in the heart. Clearly, the regulation of AR activity and by implication the role of this enzyme during myocardial ischemia-reperfusion are complex and deserve further investigation. Nevertheless, our previously published data showing that inhibition of AR by tolrestat, which inhibits both activated and non-activated AR (52), increases myocardial infarction in conscious rats provide strong evidence that AR prevents ischemic injury *in situ* under physiological concentrations of

blood gases and bloodborne inflammatory mediators and other modulators of ischemic injury.

In summary, our results show that during myocardial ischemia AR is activated by the oxidation of its cysteine residues to sulfenic acid. The activated enzyme is deactivated by a two-step process, which involves GSTP-catalyzed addition of GSH to AR-SOH followed by GRX-mediated dethiolation. This process preserves AR protein and returns it to its basal, unactivated state after a brief excursion to an activated state. These findings uncover a new adaptive/protective mechanism that is activated by oxidative stress and is then "switched off" when it is no longer needed. Additional studies are required to determine whether other protein sulfenic acids are also controlled by similar redox-regulated switches.

Acknowledgments—We are grateful to Dr. J. J. Mieyal (Case Western Reserve University) and Dr. S. V. Singh (University of Pittsburgh) for providing cDNAs for human GRX and GSTP, respectively. We thank Drs. C. Henderson and R. Wolf, University of Dundee, for providing breeding pairs of GSTP mice.

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