ANP-induced decrease of iron regulatory protein activity is independent of HO-1 induction

Alexandra K. Kiemer,1,2 Anke C. Förnges,1,2 Kostas Pantopoulos,3 Manfred Bilzer,2 Bill Andriopoulos,3 Tobias Gerwig,1,2 Silke Kenngott,2 Alexander L. Gerbes,2 and Angelika M. Vollmar1

1Department of Pharmacy, Center of Drug Research, 2Department of Medicine II, Klinikum Grosshadern, University of Munich, Munich, Germany; and 3Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec, Canada H3T 1E2

Submitted 10 December 2003; accepted in final form 9 April 2004

ANP-induced decrease of iron regulatory protein activity is independent of HO-1 induction. Am J Physiol Gastrointest Liver Physiol 287: G518–G526, 2004. First published April 15, 2004; 10.1152/ajpgi.00514.2003.—Atrial natriuretic peptide (ANP)-preconditioned livers are protected from ischemia-reperfusion injury. ANP-treated organs show increased expression of heme oxygenase (HO)-1. Because HO-1 liberates bound iron, the aim of our study was to determine whether ANP affects iron regulatory protein (IRP) activity and, thus, the levels of ferritin. Rat livers were perfused with Krebs-Henseleit buffer [±ANP, 8-bromo-cGMP (8-Br-cGMP), and tin protoporphyrin, 20 μM], stored in University of Wisconsin solution (4°C, 24 h), and reperfused (120 min). IRP activity was assessed by gel-shift assays, and ferritin, IRP phosphorylation, and PKC localization were assessed by Western blot. Control livers displayed decreased IRP activity at the end of ischemia but no change in ferritin content during ischemia and reperfusion. ANP-pretreated livers showed reduced IRP activity, an effect mimicked by 8-Br-cGMP. Ferritin levels were increased in ANP-pretreated organs. Simultaneous perfusion of livers with ANP and tin protoporphyrin did not reduce ANP-induced action, arguing against a role for HO-1 in changes in IRP activity. ANP and 8-Br-cGMP decreased membrane localization of PKC-α and PKC-ε, but this modulation of PKC seems unrelated to inhibition of IRP binding. This work shows the cGMP-mediated attenuation of IRP binding activity by ANP, which results in increased hepatic ferritin levels. This change in IRPs is independent of ANP-induced HO-1 and reduced PKC activation.

cGMP; ischemia-reperfusion injury; heat-shock proteins; hepatoprotection; hormonal preconditioning

Heme oxygenases (HO-1 and HO-2) are catabolic enzymes necessary to detoxify excess protoporphyrin IX, which is derived mainly from hemoglobin in senescent erythrocytes (2, 22). In contrast to constitutively expressed HO-2, HO-1 is an inducible enzyme. Induction of HO-1 occurs upon various stimuli, including heavy metals, reactive oxygen species (ROS), cytokines, and protoporphyrin IX, the substrate for HO. HO-1 decomposes protoporphyrin IX by cleaving its α-methylene bridge to generate equimolar amounts of CO, biliverdin-IXα, and divalent iron (47).

The HO-1-mediated liberation of bound iron leads to increased cellular ferritin levels (54, 55). Ferritin is a widely distributed and highly conserved protein; its major task is to detoxify intracellular iron (1, 49). Despite the important role of iron in the organism, unbound iron is detrimental because of its corrosive reactivity with oxygen and its potency to catalyze the production of highly reactive hydroxyl radicals via the Fenton reaction (17). Ferritin is a large multienzyme protein mainly located in the cytosol; it keeps the stored iron separated from the nucleus and other organelles. It contains 24 subunits with a molecular mass of 450 kDa (without bound iron).

The biosynthesis of ferritin is mainly controlled by a post-transcriptional, iron-dependent regulatory system based on the interaction of cytoplasmic iron regulatory proteins (IRPs) and noncoding 5′-untranslated region sequences named iron responsive elements (IREs) in the ferritin mRNA (Fig. 1) (21). The latter represent particular hairpin structures that are recognized by the trna-acting IRPs. Two closely related IRPs have been identified: IRP-1 and IRP-2. In the liver, only IRP-1 is found in relevant concentrations (50). IRE binding of IRPs occurs under conditions of iron deprivation, leading to reduced ferritin mRNA translation (21). On the other hand, IRPs are posttranslationally inactivated (IRP-1) or degraded (IRP-2) when iron supply to cells is increased. In such iron-replete cells, IRP-1 assembles a cubane 4Fe-4S cluster, converting IRP-1 to cytosolic aconitase and preventing IRE binding. Disulfide bonds formed by three cysteine residues have been identified as indispensable for anchoring the cluster and, therefore, as responsible for suppression of IRP-1 binding (56).

Because of the iron-releasing properties of HO-1, induction of this enzyme is suggested to decrease IRP binding activity, thereby increasing cellular ferritin levels.

When livers undergoing ischemia and reperfusion are pretreated with the hepatoprotective cardiovascular hormone atrial natriuretic peptide (ANP) (8, 13, 14, 27), these organs show increased expression of HO-1 (29). Because of this established effect of ANP, i.e., induction of HO-1, we hypothesized that ANP increases ferritin translation by decreasing the mRNA binding of IRP activity via an HO-1-induced release of iron. Regulation of iron metabolism during ischemia-reperfusion injury is poorly understood (48), and nothing is known about a potential influence of protective interventions. We therefore investigated the effect of ANP preconditioning on hepatic IRP activity and ferritin expression. We furthermore characterized the receptor specificity of ANP-induced effects and looked at...
the causal role of ANP-induced HO-1 in ANP-mediated effects on IRP activity.

MATERIALS AND METHODS

Materials. Rat ANP was purchased from Calbiochem/Novabiochem (Bad Soden, Germany), [α-32P]UTP (3,000 Ci/mmol) from Amersham-Pharmacia (Braunschweig, Germany), enzymes from MBI Fermentas (St. Leon Roth, Germany), and Complete from Roche Diagnostics (Mannheim, Germany). All other materials were purchased from Sigma (Taufkirchen, Germany) or VWR (Munich, Germany).

Liver perfusion. Liver perfusions were performed as previously described (13). Briefly, isolated rat livers were perfused with Krebs-Henseleit (KH) buffer blood free (“0 min”) or for 30 min before ischemia. Organs were preconditioned by addition of ANP or 8-bromo-cGMP (8-Br-cGMP) in the presence or absence of tin protoporphyrin (SnPP) to the preischemic perfusate (20 min). Livers were then kept in University of Wisconsin (UW) solution (4°C) containing ANP or 8-Br-cGMP for 24 h. After ischemia, livers were reperfused for up to 2 h. To check for potential effects of perfusion on IRP-1 activity, livers were perfused for 150 min. At the indicated times, livers were snap frozen and stored at −80°C until further analysis. Unless otherwise stated, four or five independent experiments were performed in each group. All animals received humane care. The study was registered with the local animal welfare committee.

Western blot. Liver samples were homogenized in ice-cold lysis buffer (5 mM Tris, 40 mM KCl, 1% Triton X-100, 0.1 mM PMSF, pH 7.4). Protein (400 μg) was heated to 70°C for 10 min. This procedure keeps the heat-resistant ferritin in the supernatant, whereas it denatures most proteins and allows their removal by centrifugation. Cleared supernatants were resolved on SDS-polyacrylamide gels, and blotted proteins were detected with an antiferritin antibody (Sigma).

Immunoprecipitation. Liver samples were lysed in ice-cold buffer (25 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, and Complete). Protein (500 μg) was incubated overnight with 10 μl of rabbit anti-IRP-1 antibody at 4°C. Protein A-agarose (50 μl) was added for another 2 h. Washed samples were resolved in a Laemmli system and blotted, and murine antiphosphoserine antibody (Sigma) was used to detect phosphorylated IRP-1. To check for homogeneous immunoprecipitation, blots were reprobed with IRP-1 antibody. To confirm specificity of the immunoprecipitation, 10 μl of control rabbit serum were added to the binding reaction, instead of specific anti-IRP-1 antibody.

IRP binding activity to IRE elements: RNA EMSA. An α-32P-labeled IRE probe was generated by in vitro transcription of the plasmid I-12.CAT (15, 40) using T7 RNA polymerase after linearization with XhoI. Cytoplasmic extracts were prepared in buffer containing 1% Triton X-100, 40 mM KCl, 25 mM Tris, 0.1 mM PMSF, and Complete, pH 7.4. Protein content was determined (35), and gel retardation assays were performed essentially as described elsewhere (34). The denatured probe (25,000 cpm) was added to 10 μg of cytoplasmic extract in the presence of heparin (50 μg) to avoid unspecific binding, and the reaction mixtures were incubated for 10 min. Samples were resolved in 5% nondenaturing polyacrylamide gels, and the bands were detected by phosphor imaging (Canberra-Packard, Dreieich, Germany). Recombinant IRP-1 activated by N-acetyl-l-cysteine (MBI Fermentas) served as a positive control. To control for equal protein loading, 2-mercaptoethanol was added to the reaction mixture. N-acetyl-l-cysteine and 2-mercaptoethanol reduce disulfide bonds formed by three IRP-1 cysteine residues and, thereby, maximally activate IRP-1 binding activity.

Distribution of PKC. Distribution of the PKC isoforms α, δ, and ε in hepatic tissue was determined by an immunoblotting technique exactly as described previously (4–6). Briefly, a sample of ~200 mg wet wt was excised from the anterior liver lobe, immediately transferred to ice-cold homogenization buffer (1 ml/200 mg tissue; composition (in mM): 20 Tris, 250 sucrose, 2 EGTA, 1 MgCl2, 1 PMSF, 10 β-mercaptoethanol, with 50 μM leupeptin and 25 μg/ml aprotinin, pH 7.4), and sonicated at 4°C. Cytosolic and particulate membrane fractions were separated by ultracentrifugation. Aliquots of the fractions were subjected to electrophoresis, and proteins were blotted onto Immobilon membranes (4–6). PKC bands were localized by use of affinity-purified isoenzyme-specific antibodies for PKC-α, PKC-δ, and PKC-ε, a goat anti-rabbit IgG antibody, and a chemiluminescence reagent. The bands of PKC isoenzymes were identified by their molecular weight using molecular weight markers and comparison with a rat brain cytosol preparation rich in all PKC isoforms under study. Molecular weight markers and rat brain cytosol preparations were run on each gel. The PKC bands at 80 kDa (α and δ) and 90 kDa (ε) were quantified by densitometry (NIH Image Densitometric Analysis 1.54). Results are expressed as follows: (OD of particulate fraction/total OD of cytosolic and membrane fraction) × 100 (%), where OD is optical density.
**Hepatocytes.** Hepatocytes were isolated as previously described (29). After isolation, cells were cultured for 4 h in the presence of FBS and insulin, and this medium was replaced by medium without insulin. Cells were then left untreated for another 12 h or treated with a PKC inhibitor (PKC inhibitor peptide-(19–31); Biomol, Hamburg, Germany) at 100 nM. Medium was replaced by fresh medium containing the same concentration of inhibitor after 12 h, and the cells were left untreated or treated with 100 nM PMA for the next 4 h. Cell lysis and RNA EMSA were performed as described above.

**Statistical analysis.** Values are means ± SE. The significance of differences between experimental groups was determined by one-sample t-test. *P < 0.05 was considered statistically significant. Analyses were performed with GraphPad Prism (version 3.02, Graphpad Software, San Diego, CA).

**RESULTS**

**IRP-1 activity and ferritin levels during hepatic ischemia and reperfusion.** Before determining a potential effect of ANP on IRP RNA binding activity and ferritin levels, we aimed to clarify potential changes in these iron-dependent proteins during ischemia-reperfusion. Because of the weak hepatic expression of IRP-2 (9), all our data represent activities of hepatic IRP-1. Continuous perfusion for up to 150 min had no effect on IRP-1 activity (Fig. 2A). Figure 2B shows significantly decreased IRP-1 binding activity at the end of the ischemic storage period. There were no changes in IRP-1 binding activity in the course of reperfusion. The reduced postsischemic IRP-1 binding activities did not affect ferritin levels during ischemia and reperfusion (Fig. 2C).

**ANP attenuates IRP-1 RNA binding activity and increases hepatic ferritin levels.** Preconditioning of livers with ANP for 20 min significantly reduced IRP-1 RNA binding activity (Fig. 3A). At the end of ischemia and during reperfusion, however, ANP-pretreated organs did not show altered IRP-1 levels (Fig. 3B). To ensure that altered binding activities were not due to different levels of IRP-1, samples were fully activated with mercaptoethanol. This procedure resulted in unchanged activation levels of IRP-1.

To determine the receptor specificity of ANP action on IRP-1, we assessed IRP-1 binding activity in livers preconditioned with the second messenger analog 8-Br-cGMP. 8-Br-cGMP decreased IRP-1 binding activity (Fig. 3C), whereby activation of IRP-1 with mercaptoethanol showed equal levels of IRP-1 (Fig. 3D). These data suggest that ANP mediates its inhibitory action on IRP-1 activity via the guanylate cyclase-coupled A receptor (NPR-A) (53).

Because attenuated IRP-1 RNA binding activity has been described to increase ferritin translation (21), we determined a potential effect of ANP on hepatic ferritin levels. These investigations revealed a significant increase of ferritin in ANP-treated livers at the end of the preconditioning period as well as at the end of ischemia (Fig. 4).

**Inhibition of IRP-1 RNA binding activity is independent of HO-1 activity.** Because of the potency of ANP to lead to a fast and transiently increased expression of HO-1 (29), a causal relationship between HO-1-induced iron release and reduced IRP-1 activity has been suggested (21). We therefore employed the HO-1 inhibitor SnPP, together with ANP. Interestingly, however, SnPP did not abrogate the inhibitory action of ANP on IRP-1 binding activity (Fig. 5A). SnPP alone did not affect basal IRP-1 activity. Again, activation with mercaptoethanol confirmed equal amounts of IRP-1. Besides a lack of effect on IRP-1 binding, SnPP also did not alter ANP-induced ferritin levels at the end of preconditioning or after 24 h of ischemia (Fig. 5B). These data argue against an involvement of HO-1 in the ANP-induced inactivation of IRP-1 binding activity.

**Reduction of membrane binding of PKC-α and PKC-ε by ANP.** Our data do not suggest that HO-1 is responsible for the ANP-mediated inhibition of IRP-1 binding activity. Therefore, other potential pathways of IRP-1 inactivation were investigated. It has been reported that PKC can phosphorylate IRP-1 at Ser138, leading to its activation (44, 51). Thus attenuation of PKC activity might lead to reduced IRP-1 binding. Translocation of mobile PKC isoforms from cytosol to membranes is a
key step for their activation. We therefore assessed the subcellular distribution of PKC isoforms. In control organs, the fraction of Ca\(^{2+}\)-sensitive PKC-\(\alpha\) bound to membranes was 50.1 ± 5.3%. ANP lowered membrane-bound PKC-\(\alpha\) by 39%. Similarly, 8-Br-cGMP decreased membrane-bound PKC-\(\alpha\) by 44% (Table 1). Furthermore, ANP and 8-Br-cGMP tended to reduce the membrane-bound fraction of Ca\(^{2+}\)-independent PKC-\(\epsilon\) (57.2 ± 2.6%) by 20 and 10%, respectively (Table 1). Neither ANP nor 8-Br-cGMP affected the distribution of PKC-\(\delta\) (Table 1).

**Phosphorylation of IRP-1.** Because ANP reduced PKC membrane localization, we hypothesized an altered phosphorylation state of IRP-1 on treatment with ANP. Serine phosphorylation should be assessed by detection with an antibody specifically detecting phosphorylated serine residues after immunoprecipitation of IRP-1. Control experiments confirmed the suitability of the noncommercial antibody employed to immunoprecipitate IRP-1 (Fig. 6A); there was a marked enrichment of IRP-1 after immunoprecipitation. Detection of phosphorylated serine, however, did not show a significantly

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**Fig. 3.** ANP attenuates IRP-1 activity via cGMP. Livers were perfused for 30 min in the absence (Co) or presence of 200 nM ANP (A and B) or 50 \(\mu\)M 8-bromo-cGMP (8-Br-cGMP; C and D), stored in UW solution for 24 h (±200 nM ANP or ±50 \(\mu\)M 8-Br-cGMP, 4°C), and reperfused (reperf) for 45 or 120 min. At indicated times, tissues were harvested, and cytoplasmic extracts (10 mg) were analyzed by RNA EMSA employing 25,000 cpm of an \(\alpha\)-\(\text{P}\)-labeled IRE probe. Data show representative IRE-IRP-1 complexes and densitometric analyses from ≥3 independent experiments (\(n = 3–4\) each). Significantly different from control values at respective time point: **\(P < 0.01\); ***\(P < 0.001\). Analysis of the same extracts by EMSA in the presence of 2% 2-mercaptoethanol confirmed equal loading (as indicated and data not shown).
altered phosphorylation state of livers treated with ANP compared with control organs (Fig. 6B).

Equal immunoprecipitation was confirmed by reprobing the blot with the antibody against total IRP-1. Specificity of the immunoprecipitation was demonstrated by use of control rabbit serum.

Because changes in PKC localization did not accompany changes in IRP-1 binding activity, we aimed to determine whether alteration of PKC activities can actually modify rat hepatic IRP-1 binding activity or whether this regulatory pathway is specific for HL-60 cells.

Activation of PKC can be induced by use of the phorbol ester PMA (Fig. 7). Because PMA is a strong activator of Kupffer cells and Kupffer cell activation results in the release of ROS known to modify IRP-1 activity, these experiments could not be performed in whole livers. We therefore performed respective experiments in isolated hepatocytes.

Interestingly, neither activation of PKC by PMA nor inhibition of PKC resulted in any change of IRP-1 binding. Therefore, these data do not suggest that differential phosphorylation of IRP-1 of ROS.

### Table 1. ANP attenuates membrane binding of PKC-α and PKC-ε

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<th>Control</th>
<th>ANP</th>
<th>8-Br-cGMP</th>
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<tr>
<td>PKC-α</td>
<td>50.1±5.3</td>
<td>30.5±2.8*</td>
<td>28.1±4.6*</td>
</tr>
<tr>
<td>PKC-δ</td>
<td>53.0±7.5</td>
<td>53.0±5.0</td>
<td>53.9±4.3</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>57.2±2.6</td>
<td>45.6±2.7*</td>
<td>51.5±3.3*</td>
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Values are means ± SD expressed as percent membrane-bound PKC; n = 4. Isolated perfused rat livers were preconditioned for 30 min with 200 nM atrial natriuretic peptide (ANP), 50 μM 8-bromo-cGMP (8-Br-cGMP), or carrier only (controls). After homogenization of liver tissue, cytosol and membrane fractions were separated. Distribution of PKC isoforms in liver tissue was determined by an immunoblotting technique using isoform-specific antibodies. *P < 0.05.
is responsible for ANP-mediated reduction of IRP-1 binding activity.

**DISCUSSION**

Hormonal preconditioning with ANP is a well-established experimental approach to protect against hepatic ischemia-reperfusion injury by reducing necrotic and apoptotic cell death (8, 13, 14, 16, 27). Because of the potency of ANP to rapidly reperfusion injury by reducing necrotic and apoptotic cell death experimental approach to protect against hepatic ischemia-reperfusion injury by reducing necrotic and apoptotic cell death (8, 13, 14, 16, 27). Because of the potency of ANP to rapidly induce a transient increase in expression of HO-1 (29), an enzyme known to liberate iron, we hypothesized an influence of ANP on the activity of IRPs and, subsequently, ferritin levels.

We observed that 24 h of cold ischemic storage markedly reduced IRP-1 binding activity, whereas reperfusion did not yield any further alterations. Very few data exist in the literature regarding the influence of ischemia-reperfusion on the mRNA binding activity of IRP-1: Tacchini et al. (48) reported a decreased IRP-1 activity during early reperfusion that slowly increased in the course of reperfusion. No change of IRP-1 activity was observed at the end of ischemia. However, the respective observations were made in a model of 1 h of warm ischemia, which might explain the differential time pattern of IRP-1 activity compared with our system of 24 h of cold ischemic storage.

Our data display markedly reduced IRP-1 binding activity directly at the end of ischemia, a finding confirming the observation of Wyllie et al. (58), who also reported a decrease of IRP-1 in isolated perfused rat livers on storage in UW solution for 24 h. Wyllie et al. also investigated the activity of IRP-2, which was not determined in our experimental setting.

The attenuated activity of IRP-1 seems to be independent of an induction of HO-1, because this heme-degrading and iron-liberating enzyme is induced only during reperfusion, and not at the end of ischemia (29).

Altered IRP-1 binding is more likely related to other findings of a decrease of IRP-1 activity by hypoxic conditions: Hanson and Leibold (20) demonstrated that rat hepatoma cells display reduced IRP-1 activity after 6 h of hypoxia. Such an attenuated posthypoxic IRP-1 binding activity is supported by similar reports for murine macrophages (32), human HEK 293 cells (18, 46), and mouse Hepa-1 cells (18). In contrast, only Toth et al. (52) reported increased IRP-1 binding activity in posthypoxic human cell lines (52). These seemingly contradictory findings were explained by the authors as species- and cell type-specific differences in the regulation of IRP-1 binding activity (52).

Despite a reduced IRP-1 activity during ischemia and reperfusion, we did not observe a change in hepatic ferritin levels at up to 2 h of reperfusion. Although the literature describes increased postischemic hepatic (48) and intestinal (59) ferritin levels, models of warm ischemia-reperfusion were employed, and these increases were mainly observed at later reperfusion times. Because the aim of our study was to characterize a potential link between HO-1 induced by ANP and iron metabolism in a previously well-described model of cold ischemic storage (13, 14, 27, 29), we did not investigate ANP’s action during warm ischemia-reperfusion.

Nevertheless, we observed reduced IRP-1 RNA binding activity without a change in ferritin levels. This can be explained by a reduced protein synthesis during early reperfusion (45). Although IRP-1 RNA binding is inversely correlated to ferritin translation (1, 21, 49), both components might be regulated independent of each other. It has been reported that reduced IRP-1 binding activities may even accompany reduced ferritin expression (51). This observation was made in pituitary lactotrophs after treatment with thyrotropin-releasing hormone. Ferritin was also observed to be induced independent of an IRE-IRP interaction. In this context, IL-1β has been demonstrated to induce ferritin in Hep G2 cells, whereby IRE is not responsible for this action, but other GC-rich regions in ferritin mRNA have been suggested to be involved (42). Taken together, we would like to point out that a causal relationship between effects on IRP activity and ferritin is usually anticipated but experimentally hard to prove.

Our data show for the first time the influence of ANP on proteins involved in iron homeostasis: we demonstrate a marked decrease in IRP-1 RNA binding activity in livers preconditioned with ANP (30 min). This effect was not observed at the end of ischemia; this might be explained by the fact that control organs display a markedly reduced IRP-1

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**Fig. 6. IRP phosphorylation.** Liver perfusion was performed (30 min) in the presence or absence (Co) of 200 nM ANP. A: untreated (Co) livers were homogenized, and 40 μg of liver homogenate (lane 1), 40 μl of supernatant after immunoprecipitation (lane 2), 40 μl of resolved precipitate (lane 3), or 1 μg of recombinant IRP-1 (lane 4) was analyzed for IRP-1. B: liver samples were homogenized, immunoprecipitated (IP) with an anti-IRP-1 antibody or control rabbit IgG, and blotted for phosphoserine or IRP-1.

**Fig. 7. Hepatocyte IRP-1 binding: effects of PKC activity.** Isolated rat hepatocytes were left untreated (Co) or treated with the PKC activator PMA (100 nM) and/or the PKC inhibitor peptide–(19–31) (p19–31, 100 nM), lysed, and processed for RNA EMSA.

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The alteration of IRP-1 exerted by ANP seems to have functional consequences, because hepatic ferritin levels are markedly increased at the end of the preconditioning period and after 24 h of ischemia. The very rapid increase in ferritin protein at the end of the preconditioning period might seem surprising. However, ferritin synthesis is mainly regulated on a posttranscriptional level, and very rapid changes in cellular ferritin levels have been observed, e.g., after cellular stimulation with iron (57). Also cardiac ischemic preconditioning leads to increased ferritin levels after only 25 min of ischemia (3), making a transcriptional upregulation of ferritin rather unlikely and supporting a posttranscriptional regulation by IRPs.

ANP mediates most of its biological effects via NPR-A (53). Because the cGMP analog 8-Br-cGMP mimicked ANP’s inhibitory action on IRP-1 binding activity, NPR-A seems likely to be responsible for the observed interaction with iron homeostasis. This observation might allow us to causally connect the induction of ferritin to the hepatoprotective action of ANP, because NPR-A binding seems crucial for the latter (13). Ferritin binds free iron and, thereby, prevents the formation of hydroxyl radicals via the Fenton reaction (1, 49). Because of the well-described contribution of ROS to tissue damage during reperfusion (23–26), a contribution of ferritin induction in ANP-mediated hepatoprotection seems rather likely. This notion is further supported by the potency of ANP to protect from Kupffer cell-induced oxidative cells damage (7), which might also be connected to increased ferritin levels in ANP-treated organs.

It seems of special interest that ROS are suggested not only to be produced during reperfusion but also to play a crucial role in the induction of ischemic cell damage. Rauen et al. (41) demonstrated that hypothermia induces apoptosis in hepatocytes as well as in endothelial cells. The production of ROS as well as increased free intracellular iron levels are involved in this induction of cell death (41). Interestingly, ANP was shown to protect hepatocytes from apoptotic cell death at the end of ischemia (14). This protection might be due to increased ferritin levels as observed by us.

Direct proof for a functional relation of ferritin increase by ANP with its hepatoprotective action would be of highest interest. However, because of the lack of adequate inhibitors and the early embryonic lethality of ferritin-knockout mice (11), such studies are not possible.

To determine whether the induction of HO-1 by ANP is involved in its regulatory action on IRP-1, perfusions were performed employing an inhibitor of HO-1 activity, SnPP. Because SnPP did not abrogate the inhibitory action of ANP on IRP-1 binding activity, it seems rather unlikely that the liberation of free iron by ANP-induced HO-1 is responsible for ANP’s action on IRP-1. The fact that HO-1 induction by ANP is independent of cGMP (29), together with the observation that 8-Br-cGMP does act on IRP, further supports this notion.

The RNA binding activity of IRPs cannot only be regulated by intracellular free iron levels. The activation of PKC was reported to increase IRP activities in HL-60 cells via phosphorylation at Ser138 (44). Accordingly, we assessed a potential effect of ANP on the PKC isoforms α, δ, and ε. Interestingly, ANP led to a reduced membrane localization of PKC-α and PKC-ε without affecting PKC-δ.

The data in the literature referring to an interplay between PKC isoforms and ANP are numerous. Most data describe the regulation of natriuretic peptide expression by PKC modulation (33). Activation of specific PKC isoforms by ANP have also been described: only recently, Carini et al. (10) suggested that activation of PKC-δ by ANP is responsible for its protective effect on hypoxic hepatocytes, whereas Okawa et al. (36) suggested that specific activation of PKC-ε mediates cardiac protection by ANP. In addition to such numerous works reporting an activation of PKC isoforms by ANP, there is also broad evidence for a cGMP-mediated inhibition of PKC pathways by ANP. Such reports comprise data about bovine adrenal cortex, cultured Leydig tumor cells, adrenal glomerulosa cells, and vascular smooth muscle cells (30, 31, 37–39, 43). Taken together, effects of ANP on PKC seem to largely depend on the cell type, tissue, and species investigated.

Despite a clear reduction of PKC isoforms in our rat liver model, we could not detect a functional correlation to an attenuated phosphorylation of IRP-1: control as well as ANP-treated organs displayed comparable serine phosphorylation of immunoprecipitated IRP-1. In contrast to these findings, Schäfkes and Eisenstein (44) reported a relationship between PKC activation and IRP-1 RNA binding activity in HL-60 cells. In our system, pharmacological activation or inhibition of PKC induced no changes in IRP-1 binding activity. This indicates that iron homeostasis is differentially regulated in different species and cell types (12).

In summary, our data show, for the first time, the action of ANP on proteins involved in iron homeostasis. Neither ANP-induced HO-1 induction nor PKC inhibition seems to contribute to this effect. Therefore, these observations suggest a novel mechanism by which ANP increases hepatic ferritin levels. These elevated ferritin levels might contribute to the hepatoprotective action conveyed by this cardiovascular hormone.

ACKNOWLEDGMENTS

We thank Dr. Sebastian Mueller (University of Heidelberg) for providing the IRE probe. Drs. Matthias W. Hentze (EMBL, Heidelberg, Germany) and Andrew M. Thomson (University of Western Australia, Perth, Australia) are acknowledged for providing IRP antibody. The excellent technical support of Gerald U. Denk and Cornelia Niemann is gratefully acknowledged. We thank Drs. Ulrich Beuers (University of Munich) and Andrew M. Thomson for support and numerous helpful discussions.

GRANTS

This work was supported by Deutsche Forschungsgemeinschaft Grant FOR 440/1-1.

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