Prevention of HIF-1 activation and iNOS gene targeting by low-dose cadmium results in loss of myocardial hypoxic preconditioning in the rat

Elise Belaidi,1,2 Pauline C. Beguin,1,2 Patrick Levy,1,2,3 Christophe Ribuot,1,2 and Diane Godin-Ribuot1,2

1Laboratoire HP2, Hypoxie et Physiopathologies Cardiovasculaire et Respiratoire, Institut National de la Santé et de la Recherche Médicale ERI17, Grenoble, France; 2Faculté de Médecine, Université Grenoble 1, Grenoble, France; and 3Laboratoire EFCR, Hôpital A. Michallon, CHU, Grenoble, France

Submitted 20 June 2007; accepted in final form 7 December 2007


We have described a delayed form of myocardial preconditioning induced by a single low dose of cadmium, a metal known to enhance HIF-1α degradation in vitro (10).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: D. Godin-Ribuot, Laboratoire HP2, Université Grenoble 1, Institut Jean Roget, BP 170, 38042 Grenoble Cedex 9, France (e-mail: diane.ribuot@ujf-grenoble.fr).

http://www.ajpheart.org 0363-6135/08 $8.00 Copyright © 2008 the American Physiological Society

THE REDOX-SENSITIVE TRANSCRIPTION factor hypoxia-inducible factor (HIF)-1, a key regulator of the adaptive response to hypoxia, has been suggested to be a critical mediator of late-phase myocardial preconditioning (27). It is a heterodimer consisting of HIF-1α and HIF-1β subunits. Under normoxia (N), the HIF-1α protein is degraded after hydroxylation of two proline residues by O2-dependent prolyl-4-hydroxylases (23). This targets the subunit for the von Hippel-Lindau tumor suppressor protein E3 ubiquitin ligase complex, resulting in polyubiquitination and proteasomal degradation (8). Under conditions of hypoxia, the reduced activity of prolyl-4-hydroxylases leads to HIF-1α stabilization, nuclear translocation, and dimerization to the β-subunit. HIF-1 binding to hypoxia response elements (HRE) at the DNA consensus sequence 5'-RCGTG-3’ in promoter regions drives the transcription of various genes involved in the adaptation to hypoxic-ischemic stress (27). Some of these are genes that have protein products with cardioprotective properties, with the most documented being inducible nitric oxide synthase (iNOS) (19, 24).

Cardiomyocyte protection has indeed been achieved by enhancing HIF-1α stabilization through pharmacological agents (31), expression of constitutively stable HIF-1α (11), or prolyl-4-hydroxylase gene silencing (24). More importantly, work on knockout mice has shown that HIF-1 signaling is involved in the development of hypoxia-induced delayed preconditioning (9).

Although these results point to a role for HIF-1 and iNOS in hypoxic preconditioning, no study has specifically assessed whether the interaction of these two factors is necessary to confer delayed cardioprotection in vivo, thus taking into account the complex regulatory pathways controlling the expression of target genes during the adaptation to hypoxia.

We have described a delayed form of myocardial preconditioning induced by acute intermittent hypoxia (IH) in the rat (3, 4). The aim of the present study was thus to investigate the interaction of HIF-1 with the myocardial iNOS gene in this setting using myocardial chromatin immunoprecipitation, a powerful technique that allows the direct evaluation of transcription factor binding on gene promoters (26). Furthermore, we also wanted to confirm the pivotal role of HIF-1 by evaluating whether prevention of its activation in vivo could abolish the delayed cardioprotection. For this, we have chosen a pharmacological approach and have pretreated the animals before hypoxic preconditioning with a single low dose of cadmium, a metal known to enhance HIF-1α degradation in vitro (10).

MATERIALS AND METHODS

IH model. The recommendations of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, 1996) were followed in these experiments, which were approved by the local ethics committee and the Direction des Services Vétérinaires de l’Isère, France.

Experiments were conducted on adult male Wistar rats (weight range 330–350 g) from Elevage Janvier (Le Genest St. Isles, France) housed in controlled conditions and provided with standard rat chow...
ad libitum. For exposure to IH or N, animals were housed in identical custom-made cylindrical Plexiglas chambers (length = 28 cm, diameter = 10 cm, volume = 2.2 liters) with tightly fitted lids. During 4 h, rats received repeated 1-min cycles of IH composed of 40 s of hypoxia and 20 s of N via software-driven timed solenoid valves. Hypoxia was provided by mixing pure nitrogen and compressed air in latex balloons to obtain a 10% inspired O$_2$ fraction (FIO$_2$). N consisted of administering compressed air to allow a return to 21% FIO$_2$. Animals exposed to N only received similar cycles (with the same disturbances and noise as IH) of compressed air. The FIO$_2$ level in the chambers was controlled throughout the exposure with an O$_2$ analyzer (model ML206; AD Instrument, Oxfordshire, UK). Figure 1 provides an illustration of the FIO$_2$ profile produced by the IH protocol.

Ischemia-reperfusion protocol. Twenty-four hours after IH or N, rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and treated with heparin (500 IU/kg iv). Hearts were excised, immersed in frozen Krebs-Henseleit buffer (in mM: 118.0 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 25.2 NaHCO$_3$, 11.0 glucose, and 0.5 EDTA, pH 7.4), and perfused by the aorta, in a retrograde manner at a constant pressure of 75 mmHg, according to the Langendorff method. The perfusion medium was gassed with 95% O$_2$-5% CO$_2$. The perfusion medium was FIO$_2$, pH 7.4), and perfused by the aorta, in a retrograde manner at a constant pressure of 75 mmHg, according to the Langendorff method. The perfusion medium was gassed with 95% O$_2$-5% CO$_2$. Myocardial temperature was controlled with a thermoprobe inserted into the left ventricle (LV) and maintained at 37°C. A water-filled latex balloon (no. 4; Hugo Sachs, Germany) connected to a pressure transducer was inserted into the LV and inflated to obtain a LV end-diastolic pressure (LVEDP) between 5 and 15 mmHg. The transducer was inserted into the LV and inflated to obtain a LV end-diastolic pressure (LVEDP) between 5 and 15 mmHg. The ischemia-reperfusion (I/R) protocol consisted of 20 min of stabilization, 30 min of no-flow global ischemia, and 120 min of reperfusion. Hypoxia was maintained for 40 s, after which room air was flushed into the chamber for 20 s to reestablish normoxia (21% FIO$_2$).
ANOVA. Post hoc comparisons were performed with Bonferroni t-tests. Statistical significance was set at $P < 0.05$.

RESULTS

Acute IH-induced delayed myocardial preconditioning. Acute exposure to IH induced a delayed myocardial protection characterized by a significant decrease in infarct size (15.9 ± 5.6 in IH vs. 33.8 ± 5.0% in N rats) 24 h later (Figs. 3 and 5). This was accompanied by a significant increase in CF during reperfusion (mean reperfusion value of 9.7 ± 0.4 ml·min$^{-1}$·g$^{-1}$ in IH vs. 6.7 ± 0.4 ml·min$^{-1}$·g$^{-1}$ in N rats) (Figs. 4A and 6A). In addition, the increase in LVEDP on reperfusion was significantly smaller in IH compared with N rats (mean reperfusion value of 27.3 ± 4.0 vs. 54.1 ± 3.5 mmHg) (Figs. 4B and 6B).

For the other hemodynamic parameters, there was no significant difference in the response of the various groups to I/R (Table 1). Hemodynamic values at the end of stabilization were not statistically different between the various groups studied (data not shown).

Abolition of the IH-induced cardioprotection by Ag. Selective inhibition of iNOS by Ag perfusion before I/R resulted in loss of IH-induced cardioprotection. Infarct sizes of Ag-perfused groups were increased compared with results shown in the IH group (33.1 ± 3.9 and 35.4 ± 3.9 vs. 15.9 ± 5.6% in AgN, AgIH, and IH groups, respectively) (Fig. 3). This was accompanied by a decrease in CF (mean reperfusion value of 5.8 ± 0.1 and 5.6 ± 0.1 vs. 9.7 ± 0.4 ml·min$^{-1}$·g$^{-1}$ in AgN, AgIH, and IH groups, respectively) (Fig. 4A). Finally, Ag perfusion increased LVEDP on reperfusion in both N and IH groups (mean reperfusion value of 68.3 ± 2.7 and 70.3 ± 3.0 vs. 27.3 ± 4.0 mmHg in AgN, AgIH, and IH groups, respectively) (Fig. 4B). Ag treatment did not significantly affect the other hemodynamic parameters (Table 1).

Prevention of the IH-induced cardioprotection by cadmium chloride pretreatment. Cadmium chloride administration 1 h before N or IH prevented the protective effect of IH on infarct size (27.4 ± 6.1 and 30.1 ± 5.4 vs. 15.9 ± 5.6% in CdN, CdIH, and IH rats, respectively) (Fig. 5). In addition, CF during reperfusion decreased compared with that shown in IH rats (mean reperfusion value of 6.6 ± 0.4 and 6.0 ± 0.4 vs. 9.7 ± 0.4 ml·min$^{-1}$·g$^{-1}$ in CdN, CdIH, and IH rats, respectively) (Fig. 6A). Finally, cadmium chloride pretreatment led to an increase in LVEDP during reperfusion (mean reperfusion...
other hemodynamic parameters (Table 1). Cadmium chloride treatment did not significantly affect the treated rats compared with nontreated N rats (Figs. 5 and 6). For all parameters, there was no aggravation in cadmium chloride-CdN, CdIH, and IH rats, respectively) (Fig. 6). Figure 8, shows the PCR amplification of a DNA sequence containing the HRE of the myocardial iNOS gene. The ChIP assay allows the direct evaluation of transcription factor binding on gene promoters. This technique enabled us to cross-link and immunoprecipitate HIF-1α bound on the myocardial iNOS promoter at the end of IH exposure with or without cadmium chloride pretreatment. Figure 8, A and B, shows the PCR amplification of a DNA sequence containing the HRE of the myocardial iNOS gene. PCR amplification was obtained only in the immunoprecipitated DNA from the IH group, indicating a binding of HIF-1 to the myocardial iNOS gene promoter in our cardioprotective conditions and demonstrating that cadmium chloride pretreatment specifically prevented HIF-1 targeting of the iNOS gene (Fig. 8B). Lack of amplification in mock conditions confirmed the specificity of the immunoprecipitation technique. PCR signals from DNA fragments before IP (input) show that the IP results are not due to differences in sample DNA content. Finally, lack of amplification with control PCR primers spe-
specific for intron 1 of the rat iNOS gene (3 kb from the HRE site) ensured that results were not due to genomic DNA contamination of the immunoprecipitate (data not shown).

**Myocardial iNOS gene expression after IH and its prevention by cadmium chloride pretreatment.** Western blot analysis confirmed that HIF-1 binding to promoter of the iNOS gene induced by IH resulted in a significant increase in myocardial iNOS content and that prevention of HIF-1 stabilization by cadmium chloride pretreatment abolished iNOS gene expression (Fig. 9).

**DISCUSSION**

The principal findings of this study are the following: first, it provides a direct demonstration of the interaction of HIF-1 with the iNOS gene and their role in the delayed myocardial preconditioning induced by IH in rat; second, it shows that acute administration of a low dose of cadmium can be used as a pharmacological tool to prevent HIF-1α stabilization in vivo; and third, it brings new insight into the cardiovascular consequences of cadmium exposure.

In accordance with the results of the present study, we have previously shown that acute IH induces a delayed myocardial preconditioning in the rat with a reduction of infarct size (4) and an increase in CF and a decrease in LVEDP during reperfusion (3). The improved recovery in postischemic CF brought about by this form of preconditioning could account for the myocardial tissue salvage and reduced myocardial stunning. The relationship between these parameters is underlined by the results of the present study showing their concomitant abolition by Ag and cadmium chloride pretreatment. The improvement in CF could be explained by an increase in cardiac NO availability. Indeed, many studies have demonstrated that iNOS is an essential mediator of the delayed phase of myocardial preconditioning induced by brief episodes of I/R (7), acute sustained hypoxia (32), heat stress (1), and a number of pharmacological agents (18). We have previously shown that NOS was involved in the delayed preconditioning induced by acute IH (4). In the present study, the abolition of the cardioprotective and CF-sparing effects by perfusion with the selective iNOS inhibitor Ag, before ischemia, confirms that iNOS is the isoform involved. Interestingly, the CF improvement was seen on reperfusion only because baseline values did not differ between the various groups studied. This has been reported with other forms of iNOS-related preconditioning. Thus Tosaki et al. (29) have shown that iNOS overexpression was without effect on baseline CF but that the latter was significantly increased during reperfusion in preconditioned groups. It has been proposed that the iNOS protein is induced by preconditioning in an inactive form, which requires an activation stimulus, such as that provided by ischemia, to obtain maximal enzyme activity. The activation of kinases and/or inhibitors of phosphatases by ischemia can thus promote phosphorylation of the inactive form of iNOS induced (34, 14).

The iNOS gene is one of the numerous genes upregulated by HIF-1 in the adaptive response of the cardiovascular system to hypoxia (27). In the present study, we were able to measure nuclear HIF-1 activity in tissue extracts, thus providing the first in vivo demonstration of its increase in the myocardium in...
response to hypoxia. This suggests a more important cytosolic stabilization of HIF-1α after IH since its transcriptional activity is directly related to its oxygen-mediated degradation (5). In accordance with our results, an increase in myocardial HIF-1α measured by electrophoretic mobility gel shift assay has previously been reported after simulated hypoxia with cobalt chloride in mice (31).

Our study also provides the first in vivo evidence for the targeting of the myocardial rat iNOS promoter by HIF-1α in response to IH. Other studies on the regulation of the iNOS gene by HIF-1 in the heart have been based on indirect observations using knockout iNOS mice (24, 31), overexpression of constitutively active HIF-1α (11), or prolyl-4-hydroxylase gene silencing (24). Using ChIP analysis, we demonstrate that HIF-1 directly interacts with the iNOS promoter in myocardial extracts after intermittent hypoxic preconditioning. The resulting increase in myocardial iNOS content confirmed that this interaction resulted in a transcription of the gene.

Cadmium chloride has been shown to inhibit HIF-1α stabilization in Hep3B cells by enhancing its proteasome-dependent degradation (10). In the same cell line, the ability of cadmium to suppress erythropoietin production has been linked to its inhibitory effect on HIF-1 DNA binding and erythropoietin promoter activity (16, 25). Here, we report that in vivo administration of a single low dose of cadmium before IH prevented myocardial HIF-1 activation as well as its binding to the iNOS gene, as shown by the ChIP assay, and the resultant iNOS expression. Cadmium chloride pretreatment thus abolished the delayed cardioprotection in a manner similar to that observed with the iNOS inhibitor Ag. Cadmium chloride appears to stimulate the proteasomal degradation of HIF-1α via an action on the ubiquitin system (20). Indeed, DNA microarray analysis of gene expression induced by a nonlethal dose of cadmium in human HeLa cells shows that the ubiquitin pathway is activated (33). Cadmium has been shown to promote nuclear

Fig. 8. Myocardial in vivo chromatin immunoprecipitation of HIF-1α linked to the inducible nitric oxide synthase (iNOS) hypoxia response element (HRE; shown in A). B: PCR amplification of samples after immunoprecipitation without (Mock) and with (IP) anti-HIF-1α antibody and of fragmented DNA before IP (Input). DNA was extracted from fresh hearts collected immediately after IH or N, with or without CdCl2 (1 mg/kg ip) pretreatment. C: histogram representing PCR performed on serial input dilutions confirming that amplification is proportional to DNA quantity.

Fig. 9. Western blot analysis of myocardial iNOS protein content in cytosolic extracts from hearts collected 24 h after IH or N, with or without CdCl2 (1 mg/kg ip) pretreatment. Values are means ± SE. *P < 0.05 vs. other groups.
translocation of metal-regulatory transcription factor-1 interacting with metal-responsive elements, which have been identified in promoter regions of genes involved in heavy metal homeostasis (28). Whether this adaptive response is responsible for the upregulation of the ubiquitin system and the increase in HIF-1α degradation by cadmium remains to be determined.

Cardiovascular tissues are known to be highly vulnerable to environmental chemicals and pollutants, the most well known being cadmium-containing tobacco smoke (6). Smoking is among the strongest independent predictor of premature heart disease. Second-hand smoke, recently classified as a pollutant, increases the risk of heart disease by 30%, in particular through decreased nitric oxide synthesis (2). The results of the present study, showing that a low dose of cadmium abolishes myocardial preconditioning by preventing iNOS upregulation by HIF-1, bring into light the cardiovascular risk of cadmium exposure. By linking a specific constituent of tobacco smoke such as cadmium to cardiovascular toxicity, this could help understand the molecular mechanisms by which smoking, in particular second-hand smoke, causes heart disease.

In conclusion, the abolition of the delayed myocardial preconditioning by cadmium chloride demonstrates the beneficial role of HIF-1 activation by acute IH. Whether HIF-1 also plays a role in chronic IH-related conditions, such as obstructive sleep apnea (OSA), remains to be investigated. Indeed, OSA patients are at increased risk for cardiovascular disease (30), but there is a dramatic decline in relative mortality after the age of 50 yr that could be related to activation of cardioprotective genes, such as the iNOS gene, by the nocturnal cycles of 50 yr that could be related to activation of cardioprotective genes.

ACKNOWLEDGMENTS

The authors thank Dr. Etienne Lefai for invaluable contributions to the ChIP assays, Nolwenn Miguet for help with the Western blot assays, Dr. Jean Gagnon for helpful advice and discussions, and Bruno Chapuis for the conception of the device monitoring the IH apparatus.

GRANTS

This work was supported by research grants from the Institut National de la Santé et de la Recherche Médicale, the Région Rhône-Alpes, and the Association Grenobloise des Insuffisants Respiratoires. E. Belaidi and P. C. Beguin are recipients of doctoral fellowships from the Ministère de l’Enseignement Supérieur et de la Recherche.

REFERENCES

29. Tosaki A, Maulik N, Elliott GT, Blasig IE, Engelman RM, Das DK. 
Preconditioning of rat heart with monophosphoryl lipid A: a role for nitric 
30. Wolf J, Lewicka J, Narkiewicz K. Obstructive sleep apnea: an update on 
mechanisms and cardiovascular consequences. Nutr Metab Cardiovasc 
31. Xi L, Taher M, Yin C, Salloum F, Kukreja RC. Cobalt chloride induces 
delayed cardiac preconditioning in mice through selective activation of 
HIF-1α and AP-1 and iNOS signaling. Am J Physiol Heart Circ Physiol 
32. Xi L, Tekin D, Gursoy E, Salloum F, Levasseur JE, Kukreja RC. 
Evidence that NOS2 acts as a trigger and mediator of late preconditioning 
induced by acute systemic hypoxia. Am J Physiol Heart Circ Physiol 283: 
H5–H12, 2002.
33. Yamada H, Koizumi S. DNA microarray analysis of human gene ex-
pression induced by a non-lethal dose of cadmium. Ind Health 40: 
159–166, 2002.
34. Zhao L, Weber PA, Smith JR, Comerford ML, Elliott GT. Role of 
ducible nitric oxide synthase in pharmacological “preconditioning” with 