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Effect of CRH on NO bioavailability, ROS production and antioxidant defense systems in endothelial EAhy926 cells

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Abstract
Local or 'Immune' Corticotropin-Releasing Hormone (CRH) is secreted in peripheral tissues and plays a direct immunomodulatory role as an endocrine or paracrine mediator of inflammation. The present study was undertaken to determine whether CRH affects the endothelial redox state. Accordingly, intracellular reactive oxygen species (ROS) content and peroxynitrite levels, endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) levels as well as catalase activity, superoxide dismutase (SOD) activity and glutathione (GSH) levels were measured in the presence or absence of selective CRH receptor-1 and CRH receptor-2 inhibitors in endothelial EAhy926 cells exposed *in vitro* in 10^{-7} M CRH for 2 h. CRH acting through both receptors induced a significant increase of ROS content ($p < 0.001$), catalase activity ($p < 0.001$) and SOD activity ($p < 0.001$), accompanied by a simultaneous significant decrease of eNOS activity and NO levels ($p < 0.001$), as well as a significant increase in nitrotyrosine (peroxynitrite) levels ($p < 0.05$). The data indicate that CRH may act as a regulator of pro-inflammatory mechanisms inducing adaptation of endothelial cell function to local stress.

Keywords: *CRH, reactive oxygen species, nitric oxide, eNOS, catalase, GSH, endothelial cells, inflammation*

Introduction

CRH, a 41-amino acid peptide, is known to be the predominant regulator of the neuroendocrine, autonomic and behavioural responses to stress. Apart from the hypothalamus, it has been detected in many areas of the central nervous system, where its action targets autonomous functions and behaviours that are stress-dependent [1]. Furthermore, it has been suggested that not only adrenal secreted glucocorticoids but also CRH might be directly involved in the local regulation of inflammation.

CRH, whose presence in inflammatory sites of peripheral tissues is well documented, is considered to play a direct immunomodulatory role as an endocrine or paracrine mediator of inflammation. CRH hypersecretion has been shown in human tissues undergoing inflammatory processes, including various experimental models of inflammation in rats and mice. Peripheral CRH expression was enhanced under inflammatory conditions, where concentrations of CRH were much higher in inflamed tissue fibroblasts and vascular endothelial cells than in non-inflamed tissues [2].

The actions of CRH in the regulation of homeostasis [1,3] are mediated via activation of its two known receptors, CRH receptor 1 (CRHR1) [4] and 2 (CRHR2) [5–8]. These receptors share ~ 71% amino acid sequence similarity [9] and are distinct both in their localization in CNS and periphery [10,11] and in their binding affinities for CRH. CRHRs are G protein-dependent, acting mainly through Gs alpha enhancing cAMP production. According to the literature, two specific receptor antagonists, antalarmin and astressin-2β, are used for blocking CRHR1 and CRHR2, respectively [12–15].

Endothelial cells control vascular homeostasis by generating paracrine factors that regulate vascular tone, inhibit platelet function, prevent adhesion of

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leukocytes and limit proliferation of vascular smooth muscle. The dominant factor responsible for many of these effects is the endothelium-derived nitric oxide (NO). This compound is one of the smallest and most ubiquitous messenger molecules in mammals. NO is formed from L-arginine by enzymatic action of nitric oxide synthetase (NOS). So far, three NOS isoforms have been identified using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor as well as other cofactors for NO generation [16]. Synthesis of NO by endothelial NOS (eNOS) is critical for the maintenance of vascular homeostasis due to its inhibitory actions on vasomotor tone. Endothelial dysfunction is characterized by reduced synthesis or enhanced inactivation of NO, alone or in combination, and is seen in conjunction with risk factors for cardiovascular disease [17,18].

Within this mechanism, an increased production of reactive oxygen species (ROS) is also observed. However, ROS are known to play a dual role in biological systems, being either harmful or beneficial to living systems [19]. The harmful effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants as well as by antioxidant enzymes. Such antioxidant defences are extremely important as they represent the direct removal of free radicals (prooxidants), thus providing maximal protection for biological sites. The most efficient enzymatic antioxidants include catalase and glutathione peroxidase [20].

Studies on CRH and vascular endothelial cells suggested that CRH may play a pivotal role in the regulation of vascular endothelial function under normal and pathological conditions [21]. Human endothelial cells produce CRH locally and vascular endothelium expresses both types of CRH receptors [22–24]. However, the impact of CRH on the regulation of primary antioxidant enzymes activity and ROS production of the endothelium is unknown.

The aim of the present study was to evaluate for the first time the effect of CRH on NO bioavailability and ROS content in endothelial cells. Furthermore, we studied the effect of CRH on the activities of eNOS, catalase, SOD and glutathione peroxidase. The implication of both CRH receptors was also investigated using selective CRH receptor antagonists in order to evaluate their role in the modulation of stress as well as in the maintenance of homeostasis.

Materials and methods

Reagents

Corticotropin-releasing hormone (CRH), selective antagonists for CRHR1 and CRHR2, antalarmin and astressin-2β, respectively, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium nitrite, nitrate, nitrate reductase, nicotinamide adenine dinucleotide, hydrogen peroxide, glutathione reductase, and other reagents were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media were purchased from Gibco (Gibco BRL, CA, USA).

Cell cultures

The human endothelial cells EAhy926 were kindly provided by Dr Ch. Tsatsanis (University of Crete, School of Medicine, Laboratory of Clinical Chemistry, Greece) in the means of scientific collaboration. These cells were donated to the University of Crete by Dr C. J. S. Edgell [25]. EAhy926 cells were plated in 25 cm dishes and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 10 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (purchased from Invitrogen; CA, USA), at 37°C in a 5% CO_2 atmosphere. For each experiment, confluent cells were starved in medium without FCS for 24 h. Growth medium was replaced and then CRH and its selective receptor antagonists were added at the indicated concentrations and times. All experiments were performed in triplicate and were repeated at least five times. Cell lysates were collected using a hypotonic Tris buffer, pH 7.5, in the presence of a cocktail of protease inhibitors. Protein concentrations were determined via the Bio-Rad Protein Assay (Coomassie Blue) according to the standard protocol using a spectrometer (Perkin Elmer UV/VIS Lambda 20; Massachusetts, USA). Also, culture supernatant medium was collected and both cell lysates and medium were stored at –80°C.

Measurement of nitrite (NO_2^-) and nitrate (NO_3^-) in culture supernatant media

The concentration of the nitrite (NO_2^-) and nitrate (NO_3^-) sum was determined in culture medium. The Griess reaction was repeated after the nitrate in each specimen had been enzymatically converted to nitrite. For this purpose, aliquots of culture media were incubated for 30 min at 37°C with nitrate reductase (10 mU) and nicotinamide adenine dinucleotide phosphohydrogenase (100 μM). Griess reagent (100 μL; 5% v/v H_3PO_4 with 1% w/v sulphanilic acid and 0.1% w/v N-1-napthylethylenediamine) was added to aliquots containing 80 μl of culture medium and samples were incubated for 15 min at 37°C. Addition of the reagent converted nitrite into a deep purple azo compound, the absorbance of which was measured at 540 nm by a spectrophotometric plate reader (Biotek-Instruments INC, Highland Park, NY, USA).

The concentration of the sum of nitrite and nitrate (NO) in each sample was determined by comparing the measured absorbance to a standard curve with known sodium nitrite ($NaNO_2$) concentrations. The lowest detection limit of the method was 0.2 μM. Each sample was analysed in triplicate.

eNOS activity assay

The catalytic activity of eNOS was determined by the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline. The assay was performed according to the literature [26–28]. EAhy926 cells treated with various effectors were lysed and protein lysates (25 μg) were incubated with 1 mCi/ml L-[^{3}H] arginine, in the presence of 6 mM $CaCl_2$, for 1 h at 37°C. The reaction was stopped with EDTA-HEPES buffer and scintillation fluid was added to vials. Incorporated radioactivity was measured in a liquid scintillation *β*-counter (Wallac 1409). Furthermore, a standard curve of known eNOS Units was carried out in order to determine the correlation between eNOS concentration and activity expressed in counts.

ROS measurement in cells

ROS levels were measured using 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA; Molecular Probes, Invitrogen Detection Technologies; CA, USA). EAhy926 treated cells were loaded with 10 μM H2DCF-DA for 30 min at 37°C. Cells were then trypsinized, resuspended in PBS (10^6 cells/ml) and submitted to fluorescence analysis. Fluorescence was measured at 520 nm following excitation with 488 nm light in a VersaFluor™ Fluorometer (Biorad; Hercules, CA, USA).

Quantitative determination of nitrotyrosine

Peroxynitrite in the culture supernatants was determined indirectly by measuring nitrotyrosine levels using an ELISA kit (The BIOXYTECH®, Portland, OR). According to this method, nitrotyrosine captured by a solid phase monoclonal antibody (nitrated keyhole limpet haemocyanin raised in mouse) was detected with a biotin-labelled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate that binds to the biotinylated antibody was then utilized. Tetramethylbenzidine (TMB) was used as substrate, which forms a yellow product that absorbs at 450 nm. The absorbance was measured via an ELISA reader (Biotek Instruments INC, Highland Park, NY, USA).

Catalase activity determination

Catalase activity was based on Aebi [29]. Cell lysates (20 μg) were added in 67 mM phosphate buffer, pH 7.4, and incubated at 37°C for a few minutes prior to measurement. Following this, 15 mM H_2O_2 was added and the rate of H_2O_2 reduction was measured in a quartz cuvette for 5 min at 240 nm. The experiments were performed in triplicate and were repeated five times.

Superoxide dismutase activity determination

SOD catalyses the dismutation of superoxide radical ($O_2^{-\cdot}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2). By means of Trevigen's SOD assay kit, superoxide anions convert NBT to NBT-diformazan, which absorbs light at 550 nm. SOD reduces superoxide anion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in the experimental sample. SOD activity is determined from the percentage inhibition of the rate (ΔO.D/min) of formation of NBT-diformazan. NBT-diformazan production was measured for 5 min and each sample was analysed in triplicate. The experiments were performed in triplicate and were repeated five times.

Quantitative determination of GSH amount in cell lysates

The method is based on the ability of GSH to react with DTNB and produce a yellow compound with extinction coefficient 13.6 mM^{-1} cm^{-1}, based on Chakrapani et al. [30]. Cell lysates (10 μg) were added in 67 mM phosphate buffer pH 7.95 and DTNB 0.00396 gr/ml. The optical density was measured after incubating the samples in the dark for 30 min, at 412 nm and in triplicate for each sample.

Quantitative determination of total amount of reduced (GSH) in cell lysates

Based on Shaik and Mehvar [31], GSSG was converted into GSH by incubating cell lysates at 37°C in 67 mM phosphate buffer pH 7.95, enriched with 1 mM NADH and 1 Unit glutathione reductase. The assay proceeded according to GSH determination protocol. The experiment was performed in triplicate.

Statistical analysis

The Graph Pad Instat Statistical package for Windows was used. Data are expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) with the Bonferroni post-test was used for the comparison of data and the statistical significance limit was set at $p < 0.05$.

Results

Effect of CRH on NO release

It is well established that endothelium regulates its bioavailability of nitric oxide (NO) by different factors. Besides this, endothelial cells synthesize and secrete CRH, suggesting that this peptide could regu-

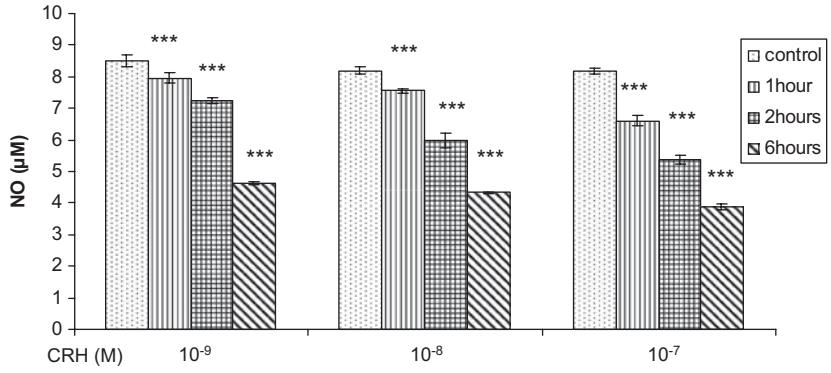

Figure 1. Time- and dose-dependent effect of CRH on NO release. After starvation for 24 h, EAhy926 cells were incubated for 1 h, 2 h and 6 h with medium alone or medium containing CRH at 10^{-9} M, 10^{-8} M and 10^{-7} M. The supernatant culture media was collected and the concentration of the nitrite (NO_2^-) and nitrate (NO_3^-) sum was determined. Data are means ± SD of the normalized data obtained in five independent experiments. All measurements were performed in triplicate in each experiment. $^{***}p < 0.001$ vs control (CTL).

late endothelial homeostasis in both an autocrine and a paracrine manner [21]. We therefore investigated in EAhy926 cells the time- and dose-dependent effect of CRH on NO release. Bearing in mind that human plasma concentration of CRH has been reported to range from 10^{-6}–10^{-11} M [32], we decided to expose EAhy926 cells to CRH concentrations 10^{-9} M, 10^{-8} M and 10^{-7} M for various time-periods (1 h, 2 h and 6 h). As shown in Figure 1, we observed that the addition of CRH significantly inhibited NO release in a dose- and time-dependant manner compared to control ($p < 0.001$). Maximal effects were observed after 6 h treatment. Subsequently, in order to determine the short-term effect of CRH, we decided to continue our experiments choosing the 2-h time-period and the CRH concentration of 10^{-7} M, since at this time and concentration we observed the maximal action (inhibition 34% at 10^{-7} M compared to 15.3% at 10^{-9} M and 27% at 10^{-8} M).

Implication of CRH receptors 1 and 2 on NO release

In order to determine which CRH-receptor sub-type (CRHR1 or CRHR2) is involved in NO release inhibition, the effect of specific antagonists for each receptor was studied. Antalarmin and astressin-2β at a concentration of 10^{-6} M were used for blocking CRHR1 and CRHR2, respectively. We observed that the inhibitory effect of CRH on NO release was abolished by both the CRHR1 and the CRHR2 antagonists (Figure 2A). Antalarmin strongly inverted the effect of CRH on NO release and increased NO levels up to 30% ($p < 0.001$). Similarly, astressin-2β tended also to restore the CRH-depended inhibition of NO release to control levels and increased NO levels up to 30% ($p < 0.001$). The inhibitory effect of CRH on NO release was completely abolished by simultaneous addition of antalarmin and astressin-2β.

Our results indicate that both CRHR1 and CRHR2 contribute to the inhibitory effect of CRH on NO release.

Regulation of eNOS activity by CRH

In order to determine whether the observed inhibitory effect of CRH on NO release was via the influence of eNOS activity, the effect of CRH on eNOS activity was studied by the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline. In EAhy926 cells, treated with CRH for 2 h at 10^{-7} M, we observed a significant inhibitory effect on eNOS activity (up to 50% compared to control) (Figure 2B). This effect was abolished when each CRH receptor antagonist was added individually at the concentration of 1 μM. CRHR1 and CRHR2 antagonists increased significantly ($p < 0.001$ and $p < 0.05$, respectively) the CRH-dependent reduction of eNOS activity (Figure 2B). The inhibitory effect of CRH on eNOS activity was also completely abolished in the presence of both CRH receptor antagonists. These findings suggest that CRH inhibits eNOS activity in endothelial EAhy926 cells and consequently provokes the decrease of NO release. This effect seems to be exerted by both receptors.

Effect of CRH on reactive oxygen species levels

Endothelium-derived NO has been identified as an anti-inflammatory molecule and inhibition of its synthesis increases vascular oxidative stress leading to inflammatory responses. Therefore, it is very likely that CRH, by inducing inhibition of NO production in EAhy926 cells, is also involved in the process of oxidative stress. As far as we know, there are no reports concerning this relationship. To investigate whether CRH affects ROS in EAhy926 cells, we performed fluorescence measurements by means of the redox-sensitive dye H2DCF-DA, a widely used intracellular ROS indicator. Under our culturing conditions, we observed that CRH increased intracellular ROS levels (up to 35% compared to the control) (Figure 3A). This effect was significantly blocked by antalarmin ($p < 0.001$) and in the presence of both CRH receptor antagonists ($p < 0.001$), but not in the presence of astressin-2β alone (Figure 3A). These data suggest

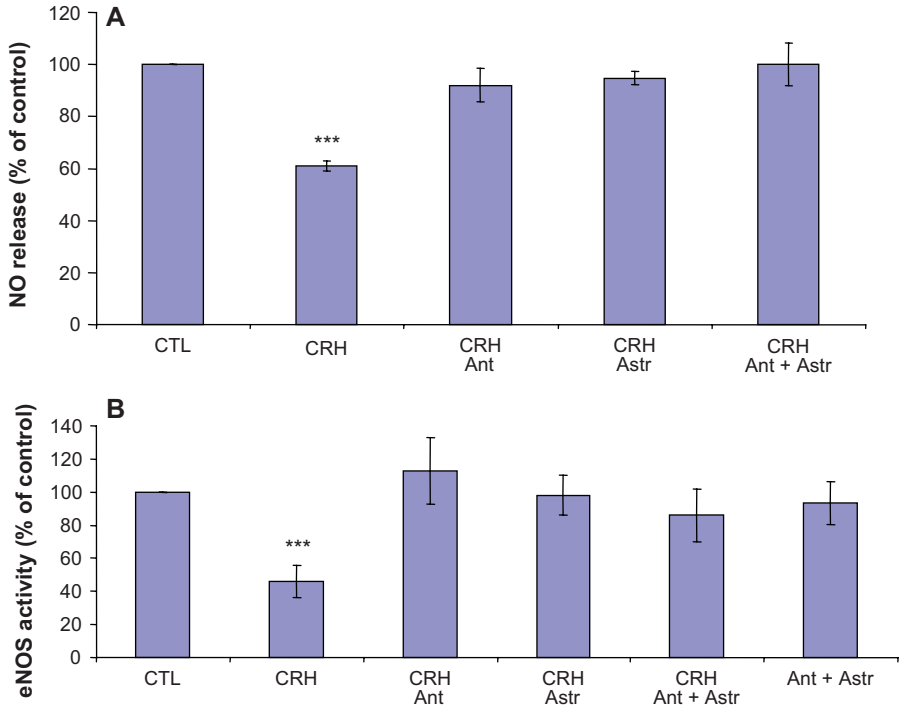

Figure 2. The effect of CRH on NO release and eNOS activity. After starvation, EAhy926 cells were incubated for 2 h in DMEM medium alone (*control*) or in the presence of CRH (10^{-7} M) or in the presence of the indicated compounds: antalarmin (*Ant*) (10^{-6} M), astressin-2β (*Astr*) (10^{-6} M). (A) The supernatant culture media was collected and the concentration of the nitrite (NO_2^-) and nitrate (NO_3^-) sum was determined. (B) Cell lysates were collected and eNOs activity was determined. Data shown are means ± SD of normalized data obtained in five independent experiments. All measurements were performed in triplicate in each experiment. $^{***}p < 0.001$ vs control (CTL).

that CRH increases the intracellular ROS content in EAhy926 cells, an effect mediated by the CRHR1 receptor.

Effect of CRH on peroxynitrite levels

Various pathways, including the formation of peroxynitrite, lead to a stable nitrotyrosine product in biological systems. Peroxynitrite formed by the reaction between two free radicals, nitric oxide and superoxide, reacts with the phenolic ring of tyrosine and forms nitrotyrosine. In order to elucidate the inhibitory effect of CRH on eNOS activity, we measured nitrotyrosine levels in the supernatant and found that reduced eNOS activity was associated with a significant increase of nitrotyrosine formation (up to 20%) compared to control ($p < 0.05$). This effect of CRH was inhibited by both CRH receptors (Figure 3B).

Effect of CRH on catalase activity

Modification of ROS rates by CRH may be due to the regulation of primary antioxidant enzymes such as catalase and glutathione peroxidase activity. Therefore, we next evaluated whether catalase activity was affected by CRH in EAhy926 cells. As shown in Figure 4A, CRH significantly up-regulated catalase activity, reaching levels even 50% higher compared to control ($p < 0.001$). Antalarmin or astressin-2β alone did not change catalase activity compared to control, whereas co-incubation of EAhy926 cells with CRH and antalarmin or astressin-2β or both reduced significantly the activity of the enzyme to levels comparable to control (Figure 4A).

These findings suggest that CRH increases catalase activity in endothelial EAhy926 cells. This effect seems to be exerted by both CRH receptors.

Effect of CRH on SOD activity

As shown in Figure 4B, CRH increased significantly (up to 25%) SOD activity compared to control ($p < 0.001$). This effect was abolished in the presence of the selective CRH receptor inhibitors, which indicates an involvement of both receptors.

Impact of CRH on GSH levels and GSH/GSH+GSSG ratio

The effect of CRH on glutathione peroxidase and glutathione reductase activity was evaluated by measuring in EAhy926 cells, in the presence or absence of CRH receptor antagonists, the levels of GSH and the ratio of GSH/GSH+GSSG. We observed that CRH decreased GSH levels compared to control, though this change did not attain statistical significance

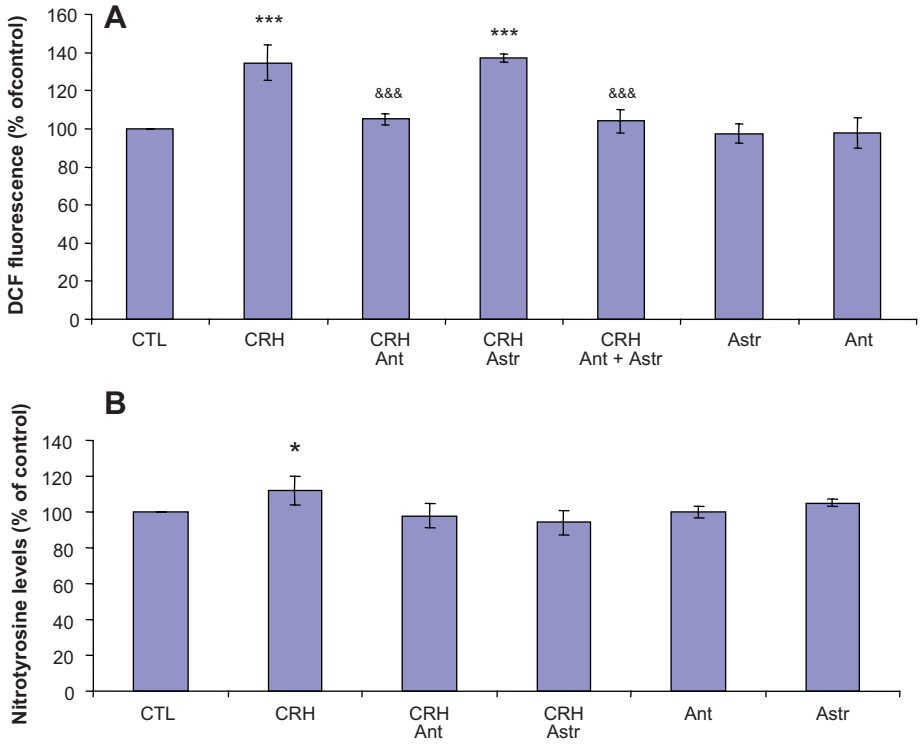

Figure 3. The effect of CRH on intracellular ROS levels and nitrotyrosine levels. After starvation, EAhy926 cells were incubated for 2 h in DMEM medium alone (*control*) or in the presence of CRH (10^{-7} M) or the presence of the indicated compounds: antalarmin (*Ant*) (10^{-6} M), astressin-2β (*Astr*) (10^{-6} M). (A) Cells were incubated with 10 μM H2DCF-DA. The relative change in mean fluorescence was calculated as the ratio of mean fluorescence for the treated to un-treated (*control*) cells. (B) The supernatant culture media was collected and the levels of nitrotyrosine were determined. Data shown are means ± SD of normalized data obtained in five independent experiments. All measurements were performed in triplicate in each experiment. $^{*}p < 0.05$ vs control, $^{***}p < 0.001$ vs control (CTL), $^{\&\&\&}p < 0.001$ vs CRH.

($p < 0.062$) (Figure 5A). Blockage of CRHR1 with antalarmin was followed by restoration of GSH to control levels. However, astressin-2β, a CRHR2 blocker, had no effect on CRH-dependent decrease of GSH. Addition of antalarmin or astressin-2β by itself had no effect on GSH levels compared to control. CRH had no impact on GSH/GSH+GSSG ratio (Figure 5B). These data may imply an effect of CRH on GSH levels mediated by CRHR1.

Discussion

The results of the present study indicate for the first time that, in endothelial EAhy926 cells, CRH induces a significant increase of cellular ROS content and of catalase activity accompanied by a simultaneous significant decrease of eNOS activity and NO levels. This effect on eNOS and catalase activity and NO release is exerted by both CRH receptors, whereas intracellular ROS content is regulated by CRHR1.

CRH has an effect on vascular endothelium as well as on smooth muscle cells [22]. It has been reported that the relaxant effect of CRH in rat aorta is predominantly endothelium-dependent [33,34]. Furthermore, it has been demonstrated that NO is involved in the vasodilatory effect of CRH in the human foetoplacental circulation [35]. However, Lei et al. [36] showed that, in the rat mesenteric artery, vasodilatation by CRH is endothelium-independent. Hence, the mechanism of action of CRH remains a subject of controversy.

In our study, eNOS activity as well as the release of NO by endothelial cell line EAhy926 was significantly reduced in response to CRH, an effect that was blocked by CRHR1 and CRHR2 selective antagonists, antalarmin and astressin-2β, respectively. Based on these results, we conclude that the short-term effect of CRH is mediated by both CRH receptors. One might speculate that CRH receptors may also initiate signalling pathways, which lead to vasoconstriction. This hypothesis is supported by a published observation [37] where, in human endothelial cells, the CRH-induced ET-1 release was mediated by CRHR2, despite the fact that NO release was not affected. This fact weakens the above theory. Our observation that eNOS activity is controlled principally by CRHR1 reinforces the theory that CRHR2 may be auxiliary in the endothelial response to CRH. CRHR2 probably possesses a more important role in a long-term NO deficiency.

ROS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems [19]. Beneficial effects of ROS

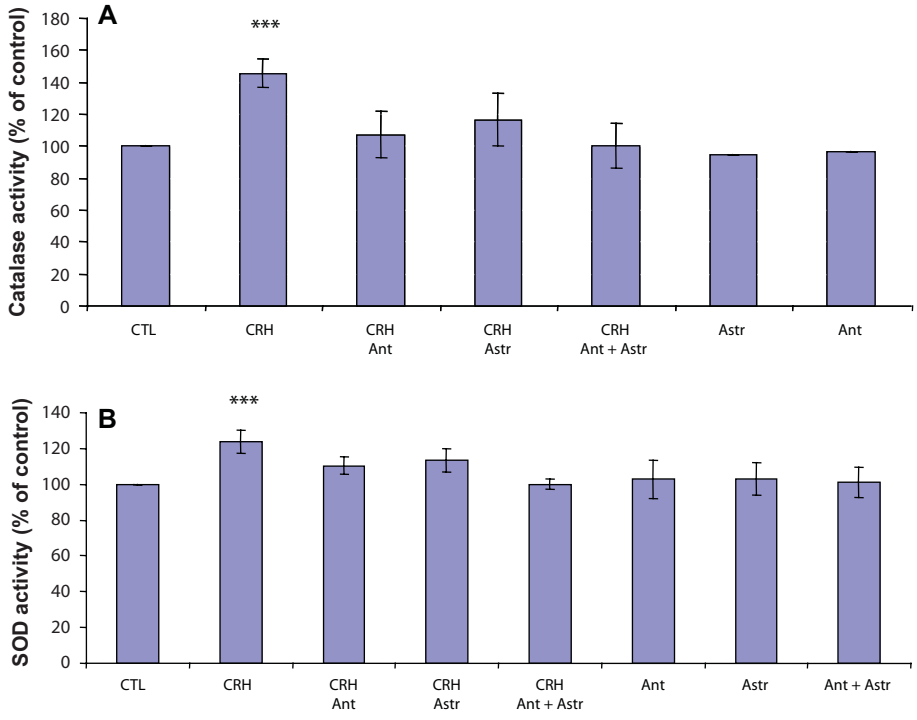

Figure 4. The effect of CRH on catalase activity and superoxide dismutase activity. After starvation, EAhy926 cells were incubated for 2 h in DMEM medium alone (*control*) or in the presence of CRH (10^{-7} M) and the indicated compounds: antalarmin (*Ant*) (10^{-6} M), astressin-2β (*Astr*) (10^{-6} M). Cell lysates were prepared and analysed for (A) catalase activity and (B) superoxide dismutase. An equal amount of proteins was used in each analysis condition. Data are shown as mean ± SD of normalized data obtained in five independent experiments. All measurements were performed in triplicate in each experiment. $^{***}p < 0.001$ vs control (CTL).

involve physiological roles in cellular responses to normoxia, as for example in defense against infectious agents and in the function of a number of cellular signalling systems. One further example of beneficial action of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids [38].

Under non-pathological conditions, eNOS produces NO by coupling with a cofactor, tetrahydrobiopterin (BH_4), using as substrate L-arginine [39]. However, in the absence of BH_4, which plays a pivotal role as a cofactor for eNOS, or L-arginine, eNOS itself can generate $O_2^{\cdot-}$ and H_2O_2 rather than NO. The deficiency in one or both of the above factors could be the reason for the decreased eNOS activity and NO levels observed in our study. However, L-arginine deficiency should be excluded because this amino acid was in sufficient concentration in the culture media. The increased peroxynitrite concentration we found indicates uncoupling of eNOS with the above-mentioned cofactor. This assumption is based on recent data, which show that peroxynitrite oxidizes BH_4, leading to a condition known as eNOS uncoupling that decreases the activity of eNOS [40].

Our results lead to the conclusion that CRH is involved in the oxidative balance of endothelial cells. We observed a significant increase of ROS intracellular levels after incubating EAhy926 cells with CRH. Interestingly, blocking CRHR1 with antalarmin reversed that ROS increase, whereas blocking CRHR2 with astressin-2β had no impact on ROS cell content. We assume that CRHR1 is the principal receptor that transduces CRH stimulation that leads to ROS elevation. Wiley and Davenport [41] suggested that peripheral CRH receptors, and especially CRHR2, may mediate a compensatory mechanism which decreases vascular tone and counterbalances the centrally mediated hypertensive effect of CRH.

The increased oxidative stress of cells triggers an immediate mobilization of the antioxidant system. In our study, catalase activity was up-regulated by CRH, which is an effect that may be linked to intracellular ROS increase at a stimulatory threshold. However, this effect of CRH was abolished when selective receptor antagonists blocked the receptors. This may indicate that both CRHR1 and CRHR2 contribute to catalase activation and protect the cell from the harmful effects of excessive ROS production.

Catalase functions together with GSH-peroxidase as an enzymatic antioxidant defense system, catalysing the decomposition of hydrogen peroxide to water to protect cells against oxygen superoxide toxicity and lipid peroxidation [42–44], while also affecting the expression of genes that influence inflammation [45]. The same trend was observed in SOD activity. Increased CRH-induced catalase and SOD activity in

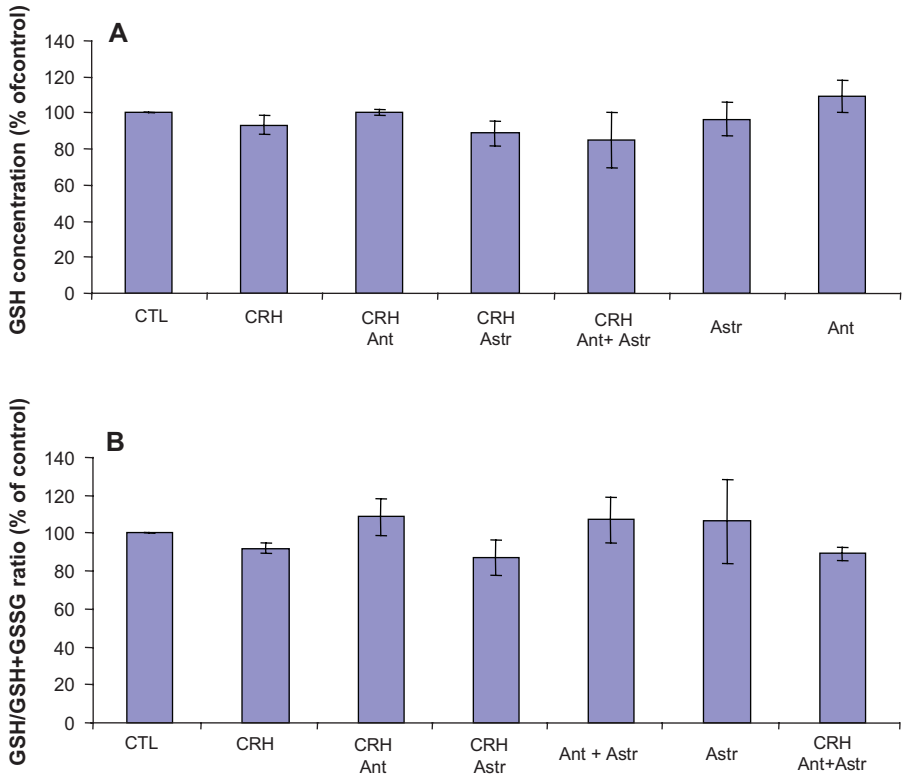

Figure 5. The effect of CRH on GSH levels and on total amount of reduced (GSH) and oxidized form (GSSG) of glutathione. After starvation, EAhy926 cells were incubated for 2 h in DMEM medium alone (*control*) or in the presence of CRH (10^{-7} M) or in the presence of the indicated compounds: antalarmin (*Ant*) (10^{-6} M), astressin-2β (Astr) (10^{-6} M). Cell lysates were prepared and analysed for (A) GSH levels and (B) the total amount of reduced (GSH) and oxidized form (GSSG) of glutathione. Equal proteins were used in each analysis condition. Data shown are means ± SD of normalized data obtained in five independent experiments. All measurements were performed in triplicate in each experiment.

our study seems to be protective through limiting the production of ROS and thereby counterbalancing the oxidative stress.

The GSH-GSSG system was not altered significantly by CRH action. This system can be described as 'the second line defence' of the antioxidant response, with SOD and catalase comprising the first enzymatic systems that remove excessive ROS. In that frame, and considering that our study concerned the early antioxidant response, it is possible that the GSH-GSSG system may need more time to express its full functional activity.

There is accumulating evidence implying that CRH has a biphasic dose-dependent effect. At increased concentrations it acts as a pro-inflammatory factor [46] and at low tissue concentration as an anti-inflammatory factor. In this context, it is important to mention that endothelial cells synthesize and secrete large amounts of CRH, suggesting that this peptide could regulate endothelial cells through CRH receptors in both an autocrine and a paracrine mode [23,37]. In our study, CRH concentration is considered to be in the range of high local concentrations, acting therefore as a pro-inflammatory agent.

Based on our data, we can conclude that CRH participates in the regulation of the endothelial cells redox balance. This short-term (acute) CRH pro-inflammatory effect may indicate an 'alert' stage that follows the homeostasis imbalance of endothelium.

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