Calcineurin expression and activity is regulated by the intracellular redox status and under hypertension in human neutrophils

Gonzalo Alba[1,*], Consuelo Santa-María[2,*], María Edith Reyes-Quiroz[1], Rajaa El Bekay[3], Isabel Geniz[4], José Martín-Nieto[5], Elizabeth Pintado[1] and **Francisco Sobrino[1,†]**

[1]Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina and [2]Departamento de Bioquímica y Biología Molecular, Universidad de Sevilla, Avenida Sánchez Pizjuán 4, E-41009 Sevilla, Spain

[3]CIBER in Physiopathology of Obesity and Nutrition (CB06/03), Instituto de Salud Carlos III, Spain, and Research Laboratory, IMABIS Foundation, Virgen de la Victoria University Hospital, Málaga, Spain

[4]Centro de Salud Guillena, Distrito Sanitario Sevilla Norte, Sevilla, Spain

[5]Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Alicante, Spain

(Correspondence should be addressed to F Sobrino; Email: fsobrino@us.es)

*(G Alba and C Santa-María contributed equally to this work)

†(F Sobrino is a senior author)

Abstract

Calcineurin (protein phosphatase 2B) (CN) comprises a family of serine/threonine phosphatases that play a pivotal role in signal transduction cascades in a variety of cells, including neutrophils. Angiotensin II (Ang II) increases both activity and *de novo* synthesis of CN in human neutrophils. This study focuses on the role that intracellular redox status plays in the induction of CN activity by Ang II. Both *de novo* synthesis of CN and activity increase promoted by Ang II were downregulated when cells were treated with L-buthionine-(S,R)-sulfoximine, an inhibitor of synthesis of the antioxidant glutathione. We have also investigated the effect of pyrrolidine dithiocarbamate and phenazine methosulfate, which are antioxidant and oxidant compounds, respectively, and concluded that the intracellular redox status of neutrophils is highly critical for Ang II-induced increase of CN expression and activity. Results obtained in neutrophils from hypertensive patients were very similar to those obtained in these cells on treatment with Ang II. We have also addressed the possible functional implication of CN activation in the development of hypertension. Present findings indicate that downregulation of hemoxygenase-1 expression in neutrophils from hypertensive subjects is likely mediated by CN, which acts by hindering translocation to the nucleus of the transcription factor *NRF2*. These data support and extend our previous results and those from other authors on modulation of CN expression and activity levels by the intracellular redox status.

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Introduction

Redox regulation of cellular processes is currently a focus of intense research (Finkel 2011). The intracellular redox status is determined by the balance between reactive oxygen species (ROS) and the cellular antioxidant defenses. Although high concentrations of ROS are catatonic, they are now known to function as signal transducing molecules at lower concentrations, modulating indirectly the activity of many enzymes and transcription factors. Classical regulation of the activity of these proteins by phosphorylation is subjected to modulation by ROS. In general, ROS increase net phosphorylation by inhibiting phosphatases and only some kinases can be directly modified by them (Paravicini & Touyz 2006).

Calcineurin (CN) is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase. Since its first proposed biological role as a putative inhibitor of calmodulin-dependent phosphodiesterase, CN has gradually revealed itself as a modulator of the immune response. CN is the main target of the immunosuppressive drugs cyclosporine A (CsA) and tacrolimus, and it is considered nowadays as a major player in Ca^{2+}-dependent eukaryotic signal transduction pathways (Lawrence *et al.* 2011). In this context, CN regulates the expression of a number of immune-response genes through dephosphorylation of a family of transcription factors known as nuclear factors of activated T cells (NF-ATs; Rusnak & Mertz 2000). CN belongs to the class of metallophosphatases containing iron and zinc in the binuclear center at their active site. During the past two decades, several studies have shed light on possible mechanisms of CN activity modulation by ROS. It has been described that superoxide is a very potent CN-inhibiting species, acting by altering the redox state of iron at the CN active site (Namgaladze *et al.* 2002). Although CN function is particularly important in brain, cartilage, and

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lymphocytes, we have shown that neutrophils also exhibit high CN expression (Carballo *et al.* 1999). Neutrophils, in addition to their classically assigned role in host defense against microbial pathogens, play a key function in the genesis of inflammatory diseases through the release of ROS (Segel *et al.* 2011).

Hypertension is considered nowadays to be an inflammatory disease, and increasing knowledge on hypertension has led to appreciation of the relevant role in this process of circulating leukocytes in general, and of neutrophils in particular (Sela *et al.* 2004, Tsukimori *et al.* 2007). Angiotensin II (Ang II) is a potent vasoconstrictor and regulator of cellular immune responses through a CN-dependent pathway (Nataraj *et al.* 1999).

Previous work from our group indicates that neutrophils are highly responsive to Ang II in the context of superoxide anion production and upregulation of CN activity and synthesis (El Bekay *et al.* 2003). In neutrophils from healthy and hypertensive subjects, we have reported that expression of the antioxidant enzyme hemoxygenase-1 (HO1 (HMOX1)) becomes induced at the mRNA and protein levels with a parallel increase in enzyme activity on treatment with prostaglandin (Alba *et al.* 2008). The present work focuses on the role that an altered intracellular redox status plays in the induction of CN activity. We have also investigated expression levels of CN in normal human neutrophils under treatment with Ang II, as well as in neutrophils from hypertensive patients, and we have analyzed the possible modulating role of CN in hypertension.

Materials and Methods

Reagents

Dextran T-500 and [γ^{32}P]ATP were obtained from Amersham Pharmacia Biotech. Ficoll-Paque was from BioWhittaker. CsA was kindly provided by Dr S F Borel (Sandoz Ltd., Basel, Switzerland). Glutathione (GSH), Ang II, L-buthionine-(S,R)-sulfoximine (BSX), diisopropyl fluorophosphate (DFP) soybean trypsin inhibitor, leupeptin, aprotinin, and goat antirabbit IgG conjugated to HRP were purchased from Sigma. The synthetic peptide used as a substrate for CN was purchased from Peninsula Laboratories. 2-Mercaptoethanol, SDS, acrylamide, *N*,*N*′-methylene-bisacrylamide, and blotting polyvinylidene difluoride (PVDF) transfer membranes were purchased from Bio-Rad. Rabbit antibovine CN IgG was kindly provided by C B Klee (National Institutes of Health, Bethesda, MD, USA). Antibodies to HO1 (sc-10789) and Nrf2 (sc-722) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Isolation and culture of human neutrophils

Human peripheral blood neutrophils were isolated from fresh heparinized blood of human donors by dextran sedimentation followed by Ficoll-Paque gradient centrifugation, and hypotonic lysis of residual erythrocytes (Carballo *et al.* 1999). Neutrophils were washed twice in Hank's balanced salt solution and then suspended at a density of 10^7 cells/ml in RPMI 1640 medium supplemented with 10% FCS plus gentamicin, penicillin, and streptomycin at 50 mg/ml each. Before all stimulations, neutrophil suspensions were pre-incubated at room temperature with 1 mM DFP (to minimize proteolysis) for 5 min (Gilbert *et al.* 2002). Neutrophils were incubated at 37 °C for the times indicated in each experiment. Under these conditions, the cells, viability ranged between 90 and 97%, as estimated by the lactic dehydrogenase release assay (Gualberto *et al.* 1998).

Subjects

Patients were eligible as hypertensive if they were >18 years old, suffered from moderate-to-high hypertension (systolic blood pressure, >140 mmHg; diastolic blood pressure, >90 mmHg; both measured for 24 h of Holter monitoring during unrestricted daily life), and they had not undergone any previous treatment with antihypertensive drugs or other compounds. Patients with diabetes mellitus, fasting glucose in serum >120 mg/dl, total cholesterol levels >240 mg/dl, or other pathologies were excluded. The criteria for normotensive subjects were to bear systolic and diastolic blood pressure values, <120 and <80 mmHg respectively. The general characteristics of all subjects used in this work are shown in Table 1. The Universidad de Sevilla Ethics Committee approved this study, and each subject gave informed consent before its undertaking.

CN activity assay

CN activity was measured as described previously (Carballo *et al.* 1999). Briefly, neutrophils (1×10^7 cells/ml) were incubated in Krebs–Ringer–HEPES buffer (KR-HEPES) containing 118 mM NaCl, 4·75 mM KCl, 1·18 mM H_2PO_4, 1·18 mM $MgSO_4$, 1·25 mM $CaCl_2$, 10 mM glucose, and 25 mM HEPES (pH 7·4), in the presence or absence of the drugs as indicated in the text. After incubation, the cells were

Table 1 General characteristics of normotensive and hypertensive subjects

	Normotensives	Hypertensives	*P* value
Sex	Five men; five women	Five men; five women	
Age (years)	40–50	40–50	NS
Weight (kg)	70·2±3·4	83·6±4·3	NS
Systolic blood pressure (mmHg)	119·3±6·3	170·1±14·0	≤0·001
Diastolic blood pressure (mmHg)	75·5±4·5	101·4±7·9	≤0·001
Body mass index (kg/m^2)	24·2±3·4	28·9±2·2	≤0·05

harvested and lysed on ice in 60 µl of lysis buffer (50 mM Tris–HCl pH 7·5, 1 mM EDTA, 0·1 mM EGTA, 1% Triton X-100, 0·5 mM dithiothreitol (DTT), 50 µg/ml soybean trypsin inhibitor, 50 µg/ml phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). A reaction mixture containing 2 µM ^{32}P-labeled phosphopeptide, 500 nM okadaic acid (added to inhibit PP-1 and PP-2 type phosphatases), 150 µM trifluoperazine, and 50 µg cell lysate protein were incubated in a total volume of 60 µl of assay buffer (20 mM Tris–HCl pH 7·5, 100 mM NaCl, 6 mM $MgCl_2$, 0·5 mM DTT, 0·1 mM $CaCl_2$, and 0·1 mg/ml BSA) for 15 min at 30 °C. Subsequently, the reaction was stopped by the addition of 0·5 ml of 100 mM potassium buffer, pH 7·0, containing 5% trichloroacetic acid. The reaction mixture was passed through a 500 µl column of activated Dowex cation-exchange resin, and free inorganic phosphate was quantitated in the eluate by scintillation counting. Data are expressed as the number of picomoles of $^{32}PO_4$ released in 15 min/mg protein.

GSH determination

Neutrophils were lysed in 315 µl of the phosphate–EDTA buffer containing 100 mM sodium phosphate (pH 8·0) and 5 mM EDTA, and 85 µl of 25% HPO_3, which was used as a protein precipitant. The total homogenate was centrifuged at 4 °C at 100 000 ***g*** for 30 min to obtain the supernatant for the assay of GSH. This was performed by a modification of the method of Hissin & Hilf (1976). To 100 µl of the 100 000 ***g*** supernatant, 565 µl Tris–HCl–EDTA buffer, containing 200 mM Tris–HCl pH 8·2 and 5 mM EDTA, was added together with 35 µl of 1 mg/ml *O*-phthalaldehyde in 100% methanol. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined at an activation wavelength of 350 nm.

Western blotting analysis of CN, HO1, and NRF2 protein levels

Total cell lysates and nuclear extracts were prepared basically as described (Carballo *et al.* 1999). Western blotting analysis of HO1 (HMOX1) protein levels was performed on total cell lysates as described (Alvarez-Maqueda *et al.* 2004). To verify even protein loading, the blots were subsequently stripped and reprobed with polyclonal antibodies against GAPDH at a 1:1000 dilution. Blots were probed with rabbit polyclonal antibodies to HO1 at a 1:1000 dilution. NRF2 levels were analyzed on nuclear extracts by using the anti-Nrf2 IgG at a 1:5000 dilution. CN levels were measured using rabbit antibovine CN IgG at a 1:1000 dilution. Band intensities were quantitated densitometrically using the Scion Image software (Scion, Inc., Frederick, MD, USA) and corrected for differences in GAPDH levels, in the case of HO1 and CN, and for differences in nuclear proteins stained with Ponceau Red, in the case of NRF2.

HO1 *mRNA levels*

Total cellular RNA extraction and reverse transcription into cDNA were performed as described previously (Alvarez-Maqueda *et al.* 2004). Real-time PCR analysis of *HO1* mRNA levels was performed using the ABI Prism 7300 sequence detection system from Applied Biosystems, under specific thermocycling conditions according to the primers used. PCRs were performed in triplicate. Each sample was also analyzed for GAPDH transcript levels to normalize for RNA input amounts. For the relative quantification of gene expression, the comparative threshold cycle method was used as described in the ABI Prism 7700 User Bulletin 2.

Glutathione peroxidase and GSH reductase activities

Glutathione peroxidase (GPx) activity was assayed with a coupled enzyme system in which oxidized glutathione (GSSG) reduction was coupled to NADPH oxidation by glutathione reductase (GR; Lawrence & Burk 1976). GR activity was determined spectrophotometrically by measuring NADPH oxidation at 340 nm (Carlberg & Mannervik 1975). All measurements were carried out in a Shimadzu 160A ultraviolet spectrophotometer using 1 ml cuvettes of 1 cm light path.

Statistical analysis

Protein expression levels are expressed in arbitrary units as the mean ± S.E.M. from a minimum number of three independent experiments. The data were subjected to ANOVA and to the Student's paired *t*-test using the Statgraphics Plus 5.0 software (Manugistic Inc., Rockville, MD, USA).

Results

Effect of Ang II and intracellular GSH levels on the induction of CN activity in human neutrophils

In a previous work, we have evidenced that Ang II increases ROS production with an associated activation of MAPK. Besides, we found that Ang II increases both activity and *de novo* synthesis of CN in human neutrophils with a maximum stimulation being obtained at a 100 nM concentration of this hormone (El Bekay *et al.* 2003). Experiments in this work were thus addressed to analyze whether the increase of CN synthesis and activity promoted by Ang II in neutrophils was modulated by the intracellular redox state. Given that reduced GSH plays an important role in control of the latter, neutrophils were treated with BSX, a specific inhibitor of γ-glutamylcysteine synthase (Cuzzocrea *et al.* 1998), in order to depress GSH levels.

Figure 1 illustrates CN activity levels measured in neutrophils previously incubated with 10 or 20 µM BSX

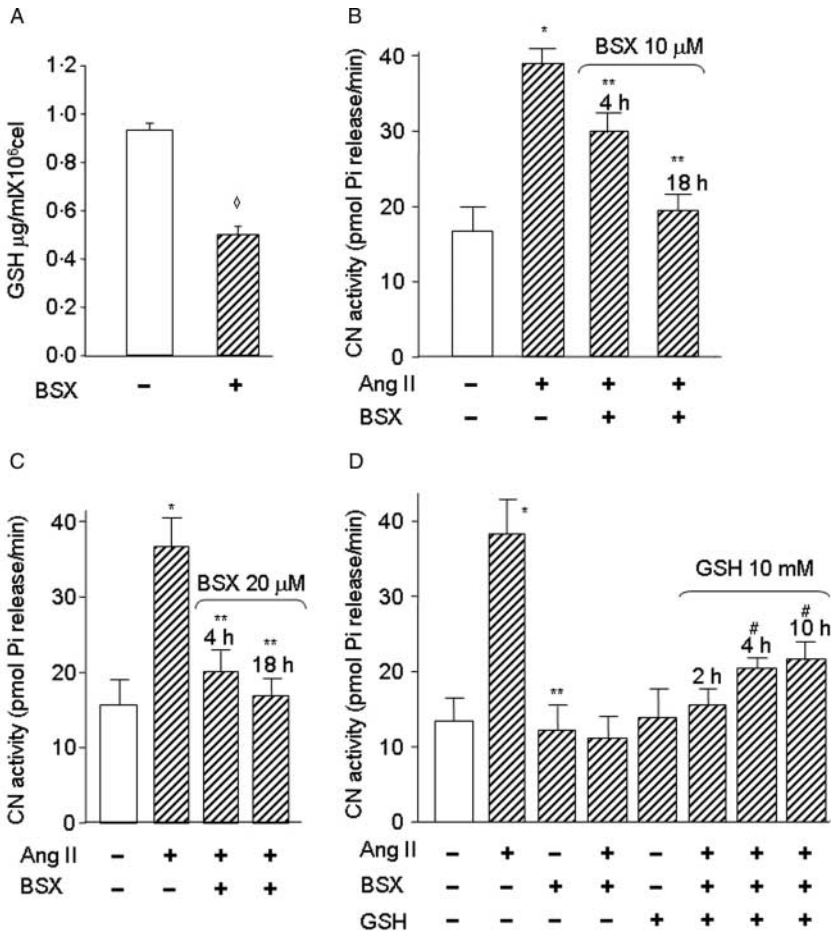

Figure 1 Depletion of endogenous GSH on treatment with BSX inhibits Ang II-induced CN activation in human neutrophils. Neutrophils (6×10^6 cells/ml) were incubated either alone or in the presence of BSX at 10 μM (B) or 20 μM (A and C) for 4 (A) or 4 or 18 h (B and C) at 37 °C, as indicated. Further, the cells were incubated in the absence or presence of 100 nM Ang II for 5 h. Some cell preparations were incubated in the presence of 10 mM GSH for 2, 4, or 10 h (D). A control including 100 nM Ang II or BSX is shown. The cells were lysed and GSH levels or CN activity was assayed as described in Materials and Methods section. Results are expressed as picomoles of Pi released/min. Mean ± S.E.M. values from three separate experiments each performed in triplicate are presented. Statistical significance: $^{\diamond}P<0{\cdot}001$, for BSX-treated vs BSX-untreated, $^{*}P<0{\cdot}001$, for Ang II-treated vs Ang II-untreated; $^{**}P<0{\cdot}01$, for BSX- and Ang II-treated vs Ang II-treated and BSX-untreated. $^{\#}P<0{\cdot}01$, for Ang II-, BSX-, and GSH-treated vs Ang II- and BSX-treated and GSH-untreated.

and subsequently treated with 100 nM Ang II. We previously tested that BSX effectively depressed GSH content in neutrophils (Fig. 1A). Treatment with this inhibitor resulted in a time-dependent loss of the ability of Ang II to enhance CN activity, this returning to basal levels (i.e. those found in the absence of Ang II) after 18 h of incubation in the presence of BSX (Fig. 1B and C). However, no *in vitro* effect of BSX alone was found on CN activity (Fig. 1D). Figure 1D illustrates that the addition of GSH to these cells was able to reverse the inhibitory effect elicited by prior BSX treatment on Ang II-mediated increase of CN activity. As shown, after 4 h of treatment with 10 mM GSH, CN activity was increased twofold by Ang II, even in the presence of BSX. By contrast, GSH addition was unable to enhance by itself Ang II-dependent CN induction.

In a previous work, we found that Ang II treatment induced the synthesis of a new CN protein (El Bekay *et al.* 2003). Here we have studied the effect of GSH depletion on Ang II-induced CN synthesis. With this purpose, we used a CN antibody, prepared from bovine CN, which for a long time has shown a very good reactivity to human CN (Aperia *et al.* 1992). By using immunological methods, we observed that the levels of both CN subunits, 19 and 59 kDa, were clearly increased 5 h after Ang II addition to neutrophils

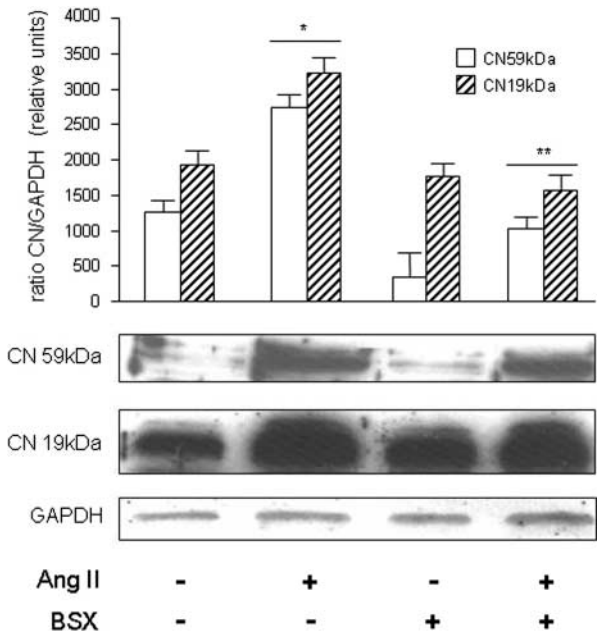

Figure 2 Depletion of GSH prevents Ang II-induced CN synthesis in human neutrophils. Neutrophils (10^7 cells/ml) were incubated either alone or in the presence of BSX at 20 μM for 18 h, and then Ang II was added at 100 nM where indicated for a further 5 h. Untreated cells were used as controls. The cells were lysed and proteins were resolved by SDS–PAGE, transferred to a PVDF membrane, and probed with a polyclonal antiserum against the A (59 kDa) and B (19 kDa) chains of CN. The bands shown are representative of a set of three independent experiments. Plotted values (mean ± S.E.M.) represent CN protein levels corrected for differences in GAPDH levels. Statistical significance: $^*P<0{\cdot}01$, for Ang II-treated vs Ang II-untreated; $^{**}P<0{\cdot}01$, for BSX- and Ang II-treated vs Ang II-treated and BSX-untreated.

(Fig. 2). It was also noticeable that Ang II-dependent *de novo* CN synthesis was decreased when the neutrophils were treated with 20 μM BSX for 20 h, but that the amount of CN protein was higher than that found in untreated cells. This fact was in contrast with the observation that BSX decreased CN activity down to basal levels, which could indirectly suggest a positive role of GSH on the catalytic center of CN (Fig. 1B).

In a new series of experiments, we analyzed whether environmental stress induced by other specific redox reagents, added to neutrophils a short time (ca. 1 h) before Ang II, could modify Ang II-dependent CN activation. First, we assayed the thiol-containing antioxidant, pyrrolidine dithiocarbamate (PDTC), and the effect we observed was opposite to that obtained with GSH, in the sense that CN activity was completely abolished after 1 h of treatment with PDTC (Fig. 3). Next, we incubated cells with an oxidant compound, phenazine methosulfate (PMS). It was found that PMS also prevented induction of CN activity by Ang II, although to a lesser extent than did PDTC (Fig. 3). These data taken together indicated that the intracellular redox status of neutrophils was highly critical for Ang II-mediated induction of CN activity.

Effect of hypertension on the induction of CN activity in human neutrophils

Hypertension is characterized by high intrarenal levels of Ang II (Navar *et al.* 2011). Thus, subsequent experiments using this hormone were carried out in human neutrophils from hypertensive patients compared with cells from healthy subjects. As shown in Fig. 4, neutrophils from hypertensive patients exhibited an increased basal expression of CN with respect to normotensive neutrophils. These high levels of CN decreased when neutrophils from hypertensive patients were incubated at 37 °C for 15 h in the absence, but not in the presence, of 100 nM Ang II. The results obtained in human neutrophils incubated with Ang II were thus very similar to those obtained in untreated neutrophils from hypertensive patients.

GSH-related enzyme levels in neutrophils from hypertensive patients

Taking into account the importance of GSH in modulation of CN activity, we studied the activity of GPx and GR, two enzymes responsible for intracellular levels of GSH in human neutrophils. The activity data obtained in hypertensive patients were normalized to those found in normotensive subjects and are shown in Fig. 5. It was observed that levels of GPx activity were ca. 22% lower in neutrophils from hypertensive patients than from healthy subjects ($P<0{\cdot}001$). By contrast, GR activity underwent a slight, but not significant, decrease in neutrophils from hypertensive patients compared with normotensive ones.

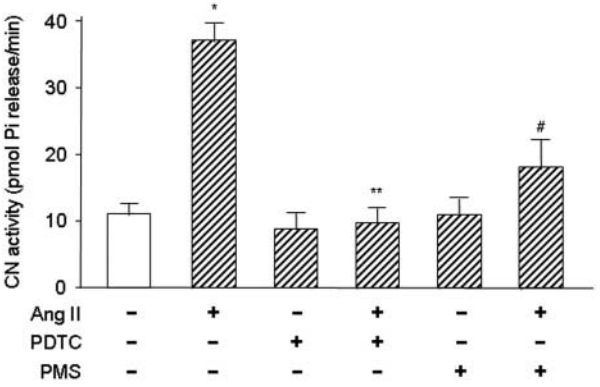

Figure 3 Effects of PDTC and PMS on Ang II-induced CN activation in human neutrophils. Neutrophils (6×10^6 cells/ml) were incubated either alone or in the presence of PDTC (100 μM) for 1 h at 37 °C or with PMS (10 μM) for 30 min. Further, the cells were incubated for 5 h in the presence of Ang II (100 nM) where indicated. The cells were then lysed and CN activity was measured. Results are expressed as picomoles of Pi released/min. Mean ± S.E.M. values from three separate experiments each performed in triplicate are presented. Statistical significance: $^*P<0{\cdot}001$, for Ang II-treated vs Ang II-untreated; $^{**}P<0{\cdot}001$, for PDTC- and Ang II-treated vs Ang-treated; $^{\#}P<0{\cdot}01$, for PMS- and Ang II-treated vs PMS-treated and Ang-untreated.

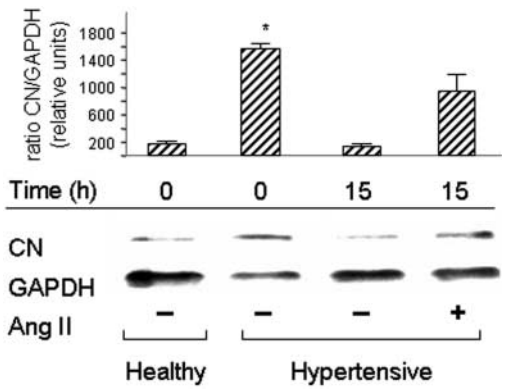

Figure 4 Levels of expression of CN in neutrophils of hypertensive patients and effect of Ang II on the expression of CN in these patients. Neutrophils from healthy subjects or hypertensive patients, as indicated, were cultured at 37 °C in RPMI medium in the absence or presence of 100 nM Ang II for 15 h. The cells were then lysed and total protein levels of CN and GAPDH were analyzed by immunoblotting. The bands shown are representative of a set of three independent experiments. Plotted values (mean ± S.E.M.) represent CN protein levels corrected for differences in GAPDH levels. Statistical significance: $*P<0{\cdot}01$, for hypertensive vs healthy.

Implication of CN in oxidative stress in hypertension

Hypertension is also characterized by an increased oxidative stress status (Drummond *et al.* 2011). In this context, HO1 is an inducible antioxidant enzyme that decreases the cellular levels of heme, a pro-oxidant group, and elevates those of bilirubin, an antioxidant (Gonzales *et al.* 2006). So, it is currently considered as a crucial enzyme to counteract intracellular oxidative stress. We have recently described that *HO1* mRNA and protein expression becomes reduced together with its enzyme activity in human normal neutrophils on their treatment with Ang II (Alba *et al.* 2008). In this work, we set to investigate whether the down-modulatory action of Ang II on HO1 expression was mediated by CN. With this purpose and because of the difficulty to use standard DNA transfection and micro-injection techniques on neutrophils *in vitro*, as they are small, short-lived, and terminally differentiated cells (Haynes & Fletcher 1990, Gaines *et al.* 2005), we used cyclosporine, a specific inhibitor of CN activity (O'Keefe *et al.* 1992, Ho *et al.* 1996). Cells thus were preincubated with CsA before subsequent treatment with Ang II and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$), the latter being a well-known inducer of HO1 expression in human lymphocytes (Alvarez-Maqueda *et al.* 2004). Figure 6A shows that Ang II prevented induction of HO1 protein expression by $15PGJ_2$. However, this negative effect of Ang II was reversed when CN was previously inhibited with CsA.

HO1 expression becomes activated by pro-oxidant reagents via translocation to the nucleus of the transcription factor nuclear factor erythroid 2-related factor 2 (*NRF2*; Alam *et al.* 1999), which has emerged as a critical regulator of the cellular response to oxidative stress. In a previous work, we found that Ang II treatment before $15dPGJ_2$ addition significantly prevented nuclear accumulation of Nrf2 (Alba *et al.* 2008). We have now studied the possible participation of CN in *NRF2* expression. As shown in Fig. 6B, it was interesting to observe that inhibition of CN also prevented the inhibition exerted by Ang II on the increase of Nrf2 nuclear levels. These results indicated that the negative effects exerted by Ang II on *HO1* expression and Nrf2 nuclear levels in human neutrophils were mediated, at least in part, through CN activation.

Finally, we compared the capacity to express HO1 by neutrophils isolated from healthy subjects and hypertensive patients. Basal levels of HO1 protein were undetectable in cells from both sources (Fig. 7). However, when neutrophils were stimulated *in vitro* with $15dPGJ_2$, synthesis of HO1 was clearly detected, although it was noteworthy that its expression levels in the presence of the prostaglandin were markedly reduced in neutrophils isolated from hypertensive patients as compared with cells from healthy subjects. By contrast, when CN was inhibited by CsA, HO1 expression was maximally induced by $15dPGJ_2$ in both groups.

Discussion

CN is a phosphatase implicated in signal transduction, whose function is particularly important in brain and lymphocytes and is susceptible to redox regulation. We have previously

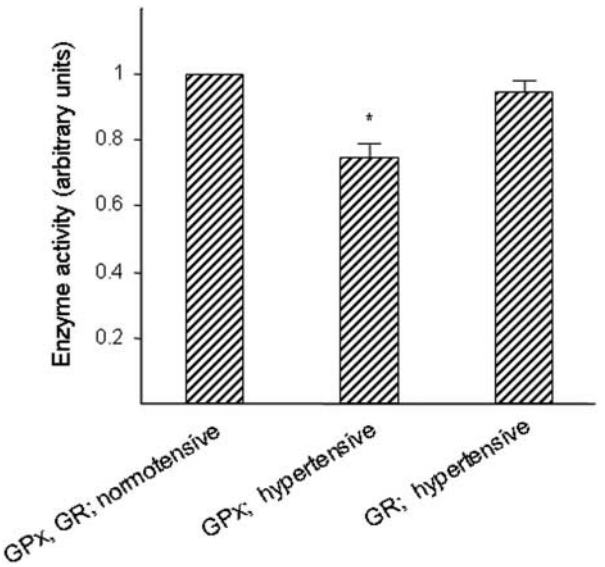

Figure 5 Effect of the hypertensive status on basal activity levels of GPx and GR in human neutrophils. Cells were lysed and extracts were used for the assay of GPX and GR enzyme activities as described in Materials and Methods sections. Values obtained in neutrophils from hypertensive patients are normalized to those from healthy donors and plotted as the mean ± S.E.M. ($n=10$). Statistical significance: $*P<0{\cdot}001$.

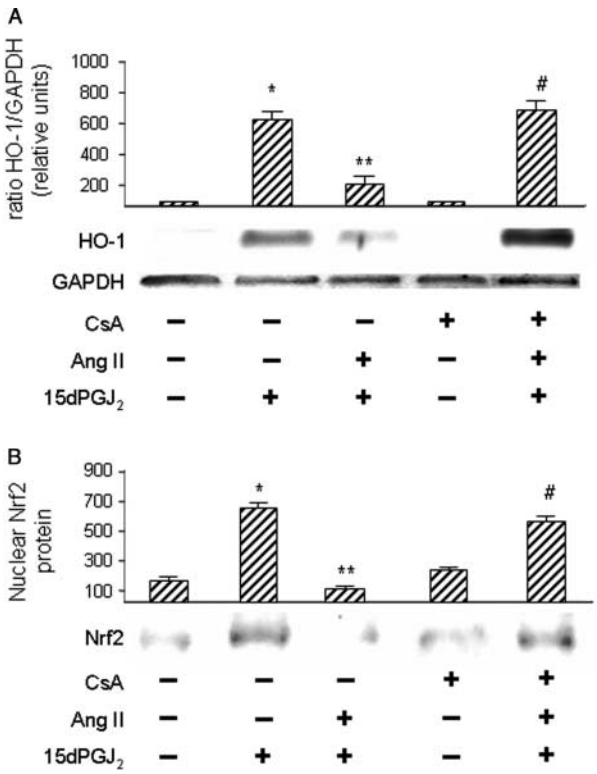

Figure 6 Effect of CN on negative modulation by Ang II of HO1 and Nrf2 nuclear translocation in human neutrophils. Neutrophils from healthy donors were grown at 37 °C in RPMI medium in the absence or presence of 0·3 g/ml CsA for 1 h (A) or 1 μg/ml CsA for 30 min (B). Then, 100 nM Ang II was added for 1·5 h (A) or 30 min (B), and thereafter, the cells were stimulated or not with 10 μM $15dPGJ_2$ for a further 5 h (A) or 30 min (B). The cells were then lysed and total proteins (A) or nuclear extracts (B) were obtained. Levels of HO1, GAPDH (A), and NRF2 (B) proteins were measured by immunoblotting analysis. The bands shown are representative of a set of three independent experiments. Plotted values (mean ± S.E.M.) represent total HO1 protein expression corrected for differences in GAPDH levels (A) or Nrf2 nuclear levels (B). Statistical significance: $^{*}P<0{\cdot}01$, for $15dPGJ_2$-stimulated vs unstimulated; $^{**}P<0{\cdot}01$, for $15dPGJ_2$-stimulated, Ang II-treated vs Ang II-untreated; $^{\#}P<0{\cdot}01$, for $15dPGJ_2$-stimulated, Ang II- and CsA-treated vs CsA-untreated.

described that CN is also present in neutrophils (Carballo *et al.* 1999), and that these cells are highly responsive to Ang II, a hormone that promotes the synthesis of superoxide anions and increases CN synthesis and activity (El Bekay *et al.* 2003). Present studies have been addressed to analyze whether CN activation promoted by Ang II in human neutrophils was modulated by the redox intracellular status.

Given that GSH is the main intracellular thiol implicated in the redox balance, we analyzed how depletion of GSH could affect the induction of CN by Ang II. With this purpose, we pretreated human neutrophils with BSX, a specific GSH synthesis inhibitor, before Ang II addition. We observed that depletion of intracellular GSH decreased the ability of Ang II to enhance CN activity and protein levels. We also investigated whether this inactivation was reversible and found that addition of GSH to neutrophils increased their CN activity.

It has been described that CN has 1–2 thiol groups essential for its full catalytic activity (Wagner & Mutus 1991). Thus, the latter can be diminished on exclusion of reducing agents, such as GSH, and the reversal of CN inhibition exerted by BSX, after incubation with GSH, may be partially due to the ability of GSH to reduce thiols. Therefore, the intracellular levels of GSH seem to be critical for Ang II-promoted induction of CN expression and activity. Other authors have described similar increases in CN activity in fibroblasts on incubation with two other thiol-reducing agents, *N*-acetyl-cysteine and lipoic acid (Sommer *et al.* 2000). The reversibility of CN inhibition by GSH is especially important in the emerging field of redox signaling. In general, reversibility is a major criterion for a particular enzyme modification to be associated with a physiological regulatory mechanism. The reversible oxidative modification of proteins, like formation of cysteine sulfenic acid or cyclic sulfenyl-amide (Salmeen *et al.* 2003), is opposed to non-specific oxidative damage to proteins, such as protein carbonyl formation or cysteine oxidation to sulfonic acid (Droge 2002). Thus, rapid recovery of CN activity after removal of an oxidative signal indicates that CN redox regulation can participate in physiological signaling.

We have also checked the effects of another antioxidant, PDTC, on CN activity. However, when this compound was added to neutrophils before Ang II addition, CN activity was drastically canceled. This compound possesses antioxidative properties by the virtue of its ability to scavenge hydroxyl radicals and superoxide anions, as well as by chelating heavy

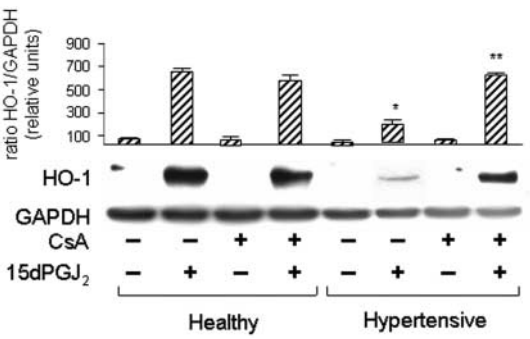

Figure 7 HO1 expression levels in neutrophils of hypertensive patients and effect of CsA on the expression of HO1 in these patients. Neutrophils from healthy subjects or hypertensive patients were cultured at 37 °C in RPMI medium in the absence or presence of CsA (0·3 g/ml) for 1 h and then stimulated or not with 10 μM $15dPGJ_2$ for further 15 h. The cells were then lysed, total proteins were obtained, and HO1 and GAPDH levels were analyzed by immunoblotting. The bands shown are representative of a set of three independent experiments. Plotted values (mean ± S.E.M.) represent HO1 protein expression levels corrected for differences in GAPDH levels. Statistical significance: $^{*}P<0{\cdot}001$, for $15dPGJ_2$-stimulated in hypertensive vs $15dPGJ_2$-stimulated in healthy; $^{**}P<0{\cdot}001$, for $15dPGJ_2$-stimulated, CsA-treated vs $15dPGJ_2$-stimulated, and CsA-untreated in hypertensive.

metals that can cause the production of hydroxyl radicals by catalyzing the Haber–Weiss reaction. Our results are in agreement with those reported by Sommer *et al.* (2000), who found that PDTC increased the activity of purified CN but inhibited most of the CN activity present in fibroblast lysates likely by chelating an exogenous metal(s) needed for its activity. By metal abstraction, PDTC inactivates copper/zinc superoxide dismutase (Kelner *et al.* 1989), a stabilizing factor in intact cells, which protects CN from inactivation (Wang *et al.* 1996). It has also been described that PDTC prevents activation of the transcription factor NF-AT in T cells, possibly by inhibiting CN phosphatase activity (Saccani *et al.* 1999).

CN belongs to the class of metallophosphatases containing iron and zinc in the binuclear center at their active site. Namgaladze *et al.* (2002) and Ghosh *et al.* (2003) have demonstrated that only under certain oxidation states in the dinuclear metal center of CN this enzyme retain catalytic activity. Indeed, Fe^{2+}/Zn^{2+} is the active form of the enzyme with very high activity, and its oxidized Fe^{3+}/Zn^{2+} form is the inactive form of the enzyme. Although, it was shown previously by Yu *et al.* (1997) that Fe^{3+}/Zn^{2} was the active form of CN.

Another approach used in the present work to expand current knowledge on regulation of CN by Ang II was based on the hypertension model, a status characterized by high levels of Ang II (Navar *et al.* 2011) and increased oxidative stress (Drummond *et al.* 2011). Besides, it is increasingly evident that the neutrophils are involved in this disease. This led us to address CN expression in neutrophils from normal and hypertensive subjects, where we obtained similar results. Next, by taking into account the importance of GSH for CN activity, we studied the activity of GPx and GR, two enzymes that modulate the intracellular levels of GSH, in neutrophils from normotensive and hypertensive subjects. This molecule has multiple roles both as an antioxidant and as a cosubstrate for the detoxification of peroxides by GPx. As a result of the action of this enzyme, GSH is oxidized to GSSG, the accumulation of which in tissues causes a variety of noxious effects. Therefore, GSSG must be reduced back to GSH in the reaction catalyzed by GR.

We found a highly significant decrease in GPx activity in neutrophils from hypertensive subjects and a slight, yet not significant, increase in GR activity. Similar results have been obtained in other cells and in animal models of hypertension (Gomez-Amores *et al.* 2006, 2007, Amirkhizi *et al.* 2010). In the hypertensive condition, the GSH/GSSG ratio is lower than in the healthy status (Gomez-Amores *et al.* 2006), probably due to associated oxidative stress. An explanation of GPx reduced activity may be that reactive aldehydes interact with the selenocysteine residue of GPx via a Michael-type addition reaction, resulting in the depletion of enzyme activity (Kondo *et al.* 2001). Another possibility may be a compensatory response by the hypertensive subjects to ameliorate GPx activity in order to maintain GSH levels. In this sense, these levels of GSH in hypertensive subjects could be sufficient to maintain CN activity.

In the present work, we have also aimed at finding a possible functional implication for the high CN activity in hypertension. In recent years, endogenously produced CO, released from heme by the activity of hemoxygenases, has been shown to exhibit vasoactive properties. These are constitutive (HO2) or inducible (HO1) isozymes able to catalyze the rate-limiting step in the metabolic conversion of pro-oxidant heme to endogenous antioxidants (biliverdin and bilirubin) and CO. HO1 expression is induced by a series of stimuli that have in common their ability to elicit oxidative stress and exhibit anti-inflammatory, anti-proliferative, anti-apoptotic, and antioxidant effects on the vasculature, in addition to protecting against atherosclerosis development (Loboda *et al.* 2008).

Our group has previously shown that HO1 synthesis is induced by $15dPGJ_2$ in neutrophils, and that HO1 expression (at the mRNA and protein levels) and activity are down-regulated in both neutrophils from healthy subjects treated with Ang II and in untreated neutrophils from hypertensive patients (Alba *et al.* 2008). As Ang II is able to induce both CN synthesis and activity in human neutrophils, we have examined in this work the possibility that the Ang II effect observed on HO1 might be mediated by CN. We found that inhibition of CN by CsA did abolish the effect of Ang II on HO1 synthesis, which allowed us to infer that Ang II exerts its effect on HO1 expression in a CN-mediated fashion. It is noteworthy that results similar to those found in Ang II-treated neutrophils were obtained in cells from hypertensive patients. The expression of HO1 and/or the application of exogenous CO have been shown to confer protection in several models of cardiovascular injury or disease, including hypertension and atherosclerosis, and in HO1 deficiency it has been related to extensive endothelial damage (Ryter *et al.* 2006).

The main transcription factor responsible for HO1 synthesis is *NRF2*. When CN was inhibited by CsA, a decrease in Nrf2 nuclear translocation was found in this work. We can thus infer that CN prevents HO1 induction by decreasing the translocation to the nucleus of the transcription factor *NRF2*. The regulation of *NRF2* is currently an issue of great interest. It has been proposed that the Nrf2 system may be a potential target for the development of indirect antioxidants aimed at the prevention/treatment of a wide array of human diseases (Jung & Kwak 2010). Dissociation of Nrf2 from Keap1 is the primary mechanism for Nrf2 translocation. The increase in the cellular oxidative status provokes the disruption of Nrf2–Keap1 interaction and thereby increases nuclear translocation of Nrf2. It has been demonstrated that inducers of *NRF2*-dependent transcription inhibit Keap1-dependent Nrf2 degradation exerted by the ubiquitin-proteasome pathway (Zhang & Hannink 2003). In mouse peritoneal macrophage cells an electrophilic agent, diethylmaleate, has been shown to facilitate nuclear accumulation of Nrf2 by suppressing

Keap1 activity (Itoh *et al*. 2003). How CN may hinder Nrf2 nuclear translocation in our neutrophil preparations is unknown. It can be speculated that CN might affect the redox status of Keap 1 and thereby decrease its dissociation from Nrf2 or promote its degradation.

Finally, we have also compared the capacity to express HO1 by neutrophils isolated from healthy subjects and hypertensive patients. HO1 expression in the presence of the inducer $15dPGJ_2$ was markedly lower in neutrophils obtained from hypertensive patients than in cells from healthy subjects. When CN was inhibited with CsA, HO1 expression was established in both groups.

In summary, the intracellular redox status of neutrophils is highly critical for Ang II-mediated induction of CN activity. These results support and extend our previous results and those of other authors on the modulation of CN activity by redox factors and its implication in the hypertension status.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM & Cook JL 1999 Nrf2, a cap'n'collar transcription factor, regulates induction of the heme oxygenase-1 gene. *Journal of Biological Chemistry* **274** 26071–26078. (doi:10.1074/jbc.274.37.26071)

Alba G, El Bekay R, Chacon P, Reyes ME, Ramos E, Olivan J, Jimenez J, Lopez JM, Martin-Nieto J, Pintado E *et al*. 2008 Heme oxygenase-1 expression is down-regulated by angiotensin II and under hypertension in human neutrophils. *Journal of Leukocyte Biology* **84** 397–405. (doi:10.1189/jlb.0108035)

Alvarez-Maqueda M, El Bekay R, Alba G, Monteseirin J, Chacon P, Vega A, Martin-Nieto J, Bedoya FJ, Pintado E & Sobrino F 2004 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induces heme oxygenase-1 gene expression in a reactive oxygen species-dependent manner in human lymphocytes. *Journal of Biological Chemistry* **279** 21929–21937. (doi:10.1074/jbc.M400492200)

Amirkhizi F, Siassi F, Djalali M & Foroushani AR 2010 Assessment of antioxidant enzyme activities in erythrocytes of pre-hypertensive and hypertensive women. *Journal of Research in Medical Sciences* **15** 270–278.

Aperia A, Ibarra F, Svensson LB, Klee C & Greengard P 1992 Calcineurin mediates alpha-adrenergic stimulation of Na^+,K(+)-ATPase activity in renal tubule cells. *PNAS* **89** 7394–7397. (doi:10.1073/pnas.89.16.7394)

Carballo M, Marquez G, Conde M, Martín-Nieto J, Monteseirin J, Conde J, Pintado E & Sobrino F 1999 Characterization of calcineurin in human neutrophils. Inhibitory effect of hydrogen peroxide on its enzyme activity and on NF-κB DNA binding. *Journal of Biological Chemistry* **274** 93–100. (doi:10.1074/jbc.274.1.93)

Carlberg I & Mannervik B 1975 Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *Journal of Biological Chemistry* **250** 5475–5480.

Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL & Szabo C 1998 Effect of L-buthionine-(S,R)-sulphoximine, an inhibitor of γ-glutamylcysteine synthetase on peroxynitrite- and endotoxic shock-induced vascular failure. *British Journal of Pharmacology* **123** 525–537. (doi:10.1038/sj.bjp.0701612)

Droge W 2002 Free radicals in the physiological control of cell function. *Physiological Reviews* **82** 47–95. (doi:10.1152/physrev.00018.2001)

Drummond GR, Selemidis S, Griendling KK & Sobey CG 2011 Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nature Reviews. Drug Discovery* **10** 453–471. (doi:10.1038/nrd3403)

El Bekay R, Alvarez M, Monteseirin J, Alba G, Chacon P, Vega A, Martín-Nieto J, Jimenez J, Pintado E, Bedoya FJ *et al*. 2003 Oxidative stress is a critical mediator of the angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF-κB. *Blood* **102** 662–671. (doi:10.1182/blood-2002-09-2785)

Finkel T 2011 Signal transduction by reactive oxygen species. *Journal of Cell Biology* **194** 7–15. (doi:10.1083/jcb.201102095)

Gaines P, Chi J & Berliner N 2005 Heterogeneity of functional responses in differentiated myeloid cell lines reveals EPRO cells as a valid model of murine neutrophil functional activation. *Journal of Leukocyte Biology* **77** 669–679. (doi:10.1189/jlb.1004567)

Ghosh MC, Wang X, Li S & Klee C 2003 Regulation of calcineurin by oxidative stress. *Methods in Enzymology* **366** 289–304.

Gilbert C, Rollet-Labelle E & Naccache PH 2002 Preservation of the pattern of tyrosine phosphorylation in human neutrophil lysates. II. A sequential lysis protocol for the analysis of tyrosine phosphorylation-dependent signalling. *Journal of Immunological Methods* **261** 85–101. (doi:10.1016/S0022-1759(01)00553-1)

Gomez-Amores L, Mate A, Revilla E, Santa-Maria C & Vazquez CM 2006 Antioxidant activity of propionyl-L-carnitine in liver and heart of spontaneously hypertensive rats. *Life Sciences* **78** 1945–1952. (doi:10.1016/j.lfs.2005.08.023)

Gomez-Amores L, Mate A, Miguel-Carrasco JL, Jimenez L, Jos A, Camean AM, Revilla E, Santa-Maria C & Vazquez CM 2007 L-carnitine attenuates oxidative stress in hypertensive rats. *Journal of Nutritional Biochemistry* **18** 533–540. (doi:10.1016/j.jnutbio.2006.10.004)

Gonzales S, Perez MJ, Perazzo JC & Tomaro ML 2006 Antioxidant role of heme oxygenase-1 in prehepatic portal hypertensive rats. *World Journal of Gastroenterology* **12** 4149–4155.

Gualberto A, Marquez G, Carballo M, Youngblood GL, Hunt SW III, Baldwin AS & Sobrino F 1998 p53 transactivation of the HIV-1 long terminal repeat is blocked by PD 144795, a calcineurin-inhibitor with anti-HIV properties. *Journal of Biological Chemistry* **273** 7088–7093. (doi:10.1074/jbc.273.12.7088)

Haynes AP & Fletcher J 1990 Neutrophil function tests. *Bailliere's Clinical Haematology* **3** 871–887. (doi:10.1016/S0950-3536(05)80139-8)

Hissin PJ & Hilf R 1976 A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry* **74** 214–226. (doi:10.1016/0003-2697(76)90326-2)

Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, Nourse J & Crabtree GR 1996 The mechanism of action of cyclosporin A and FK506. *Clinical Immunology and Immunopathology* **80** S40–S45. (doi:10.1006/clin.1996.0140)

Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T & Yamamoto M 2003 Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to Cells* **8** 379–391. (doi:10.1046/j.1365-2443.2003.00640.x)

Jung KA & Kwak MK 2010 The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules* **15** 7266–7291. (doi:10.3390/molecules15107266)

Kelner MJ, Bagnell R, Hale B & Alexander NM 1989 Inactivation of intracellular copper–zinc superoxide dismutase by copper chelating agents without glutathione depletion and methemoglobin formation. *Free Radical Biology & Medicine* **6** 355–360. (doi:10.1016/0891-5849(89)90079-8)

Kondo M, Oya-Ito T, Kumagai T, Osawa T & Uchida K 2001 Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. *Journal of Biological Chemistry* **276** 12076–12083. (doi:10.1074/jbc.M009630200)

Lawrence RA & Burk RF 1976 Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and Biophysical Research Communications* **71** 952–958. (doi:10.1016/0006-291X(76)90747-6)

Lawrence MC, Naziruddin B, Levy MF, Jackson A & McGlynn K 2011 Calcineurin/nuclear factor of activated T cells and MAPK signaling induce TNF-{alpha} gene expression in pancreatic islet endocrine cells. *Journal of Biological Chemistry* **286** 1025–1036. (doi:10.1074/jbc.M110.158675)

Loboda A, Jazwa A, Grochot-Przeczek A, Rutkowski AJ, Cisowski J, Agarwal A, Jozkowicz A & Dulak J 2008 Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities. *Antioxidants & Redox Signaling* **10** 1767–1812. (doi:10.1089/ars.2008.2043)

Namgaladze D, Hofer HW & Ullrich V 2002 Redox control of calcineurin by targeting the binuclear Fe(2+)-Zn(2+) center at the enzyme active site. *Journal of Biological Chemistry* **277** 5962–5969. (doi:10.1074/jbc.M111268200)

Nataraj C, Oliverio MI, Mannon RB, Mannon PJ, Audoly LP, Amuchastegui CS, Ruiz P, Smithies O & Coffman TM 1999 Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *Journal of Clinical Investigation* **104** 1693–1701. (doi:10.1172/JCI7451)

Navar LG, Prieto MC, Satou R & Kobori H 2011 Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension. *Current Opinion in Pharmacology* **11** 180–186. (doi:10.1016/j.coph.2011.01.009)

O'Keefe SJ, Tamura J, Kincaid RL, Tocci MJ & O'Neill EA 1992 FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357** 692–694. (doi:10.1038/357692a0)

Paravicini TM & Touyz RM 2006 Redox signaling in hypertension. *Cardiovascular Research* **71** 247–258. (doi:10.1016/j.cardiores.2006.05.001)

Rusnak F & Mertz P 2000 Calcineurin: form and function. *Physiological Reviews* **80** 1483–1521.

Ryter SW, Alam J & Choi AM 2006 Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiological Reviews* **86** 583–650. (doi:10.1152/physrev.00011.2005)

Saccani S, Saccani A, Varesio L, Ghosh P, Young HA & Sica A 1999 Divergent effects of dithiocarbamates on AP-1-containing and AP-1-less NFAT sites. *European Journal of Immunology* **29** 1194–1201. (doi:10.1002/(SICI)1521-4141(199904)29:04<1194::AID-IMMU1194>3.0.CO;2-0)

Salmeen A, Andersen JN, Myers MP, Meng TC, Hinks JA, Tonks NK & Barford D 2003 Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423** 769–773. (doi:10.1038/nature01680)

Segel GB, Halterman MW & Lichtman MA 2011 The paradox of the neutrophil's role in tissue injury. *Journal of Leukocyte Biology* **89** 359–372. (doi:10.1189/jlb.0910538)

Sela S, Mazor R, Amsalam M, Yagil C, Yagil Y & Kristal B 2004 Primed polymorphonuclear leukocytes, oxidative stress, and inflammation antecede hypertension in the Sabra rat. *Hypertension* **44** 764–769. (doi:10.1161/01.HYP.0000144480.10207.34)

Sommer D, Fakata KL, Swanson SA & Stemmer PM 2000 Modulation of the phosphatase activity of calcineurin by oxidants and antioxidants *in vitro*. *European Journal of Biochemistry/FEBS* **267** 2312–2322. (doi:10.1046/j.1432-1327.2000.01240.x)

Tsukimori K, Nakano H & Wake N 2007 Difference in neutrophil superoxide generation during pregnancy between preeclampsia and essential hypertension. *Hypertension* **49** 1436–1441. (doi:10.1161/HYPERTENSIONAHA.106.086751)

Wagner J & Mutus B 1991 Transient activation of calcineurin during thiol modification. *Second Messengers and Phosphoproteins* **13** 199–215.

Wang X, Culotta VC & Klee CB 1996 Superoxide dismutase protects calcineurin from inactivation. *Nature* **383** 434–437. (doi:10.1038/383434a0)

Yu L, Golbeck J, Yao J & Rusnak F 1997 Spectroscopic and enzymatic characterization of the active site dinuclear metal center of calcineurin: implications for a mechanistic role. *Biochemistry* **36** 10727–10734. (doi:10.1021/bi970519g)

Zhang DD & Hannink M 2003 Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Molecular and Cellular Biology* **23** 8137–8151. (doi:10.1128/MCB.23.22.8137-8151.2003)

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