Reduced to oxidized glutathione ratios and oxygen sensing in calf and rabbit carotid body chemoreceptor cells

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1. The aim of this work was to test the redox hypotheses of O_2 chemoreception in the carotid body (CB). They postulate that hypoxia alters the levels of reactive oxygen species (ROS) and the ratio of reduced to oxidized glutathione (GSH/GSSG), causing modifications to the sulfhydryl groups/disulfide bonds of K^+ channel proteins, which leads to the activation of chemoreceptor cells.

2. We found that the GSH/GSSG ratio in normoxic calf CB (30.14 ± 4.67; $n = 12$) and hypoxic organs (33.03 ± 6.88; $n = 10$), and the absolute levels of total glutathione (0.71 ± 0.07 nmol (mg tissue)$^{-1}$, normoxia *vs.* 0.76 ± 0.07 nmol (mg tissue)$^{-1}$, hypoxia) were not statistically different.

3. *N*-Acetylcysteine (2 mM; NAC), a precursor of glutathione and ROS scavenger, increased normoxic glutathione levels to 1.03 ± 0.06 nmol (mg tissue)$^{-1}$ ($P < 0.02$) and GSH/GSSG ratios to 59.05 ± 5.05 ($P < 0.001$).

4. NAC (20 μM–10 mM) did not activate or inhibit chemoreceptor cells as it did not alter the normoxic or the hypoxic release of ^{3}H-catecholamines (^{3}H-CAs) from rabbit and calf CBs whose CA deposits had been labelled by prior incubation with the natural CA precursor ^{3}H-tyrosine.

5. NAC (2 mM) was equally ineffective in altering the release of ^{3}H-CAs induced by stimuli (high external K^+ and ionomycin) that bypass the initial steps of the hypoxic cascade of activation of chemoreceptor cells, thereby excluding the possibility that the lack of effect of NAC on normoxic and hypoxic release of ^{3}H-CAs results from a concomitant alteration of Ca^{2+} channels or of the exocytotic machinery.

6. The present findings do not support the contention that O_2 chemoreception in the CB is linked to variations in the GSH/GSSG quotient as the redox models propose.

The carotid bodies (CBs) are arterial chemoreceptors activated by hypoxia and acidosis that initiate systemic responses (mostly respiratory) aimed at restoring blood P_{O_2} and P_{CO_2}/pH. Chemoreceptor cells of the CB, which are synaptically connected with the sensory fibres of the carotid sinus nerve, detect blood gases and respond to hypoxia and acidosis with an increase in the rate of release of neurotransmitters (e.g. catecholamines, CAs). An increase in the activity of the carotid sinus nerve and systemic responses follow (Gonzalez *et al.* 1994).

A fundamental aspect of chemoreceptor cell physiology, shared by pulmonary artery smooth muscle cells and erythropoietin-producing cells, is their capacity to become activated at an arterial P_{O_2} of 70–75 mmHg, when blood O_2 content is $>90\%$ (Reeves *et al.* 1979; Gonzalez, 1998). The activity of these cells increases with increasing hypoxia, and to maintain their activity they exhibit high hypoxic metabolic rates and ATP levels (Verna *et al.* 1990; Obeso *et al.* 1993). The threshold to hypoxia and the resulting systemic responses make these cells the origin of feedback loops which maintain tissue O_2 levels in the normal range when the available P_{O_2} is low (e.g. at high altitude; Richalet, 1997; Gonzalez, 1998).

Reactive oxygen species (ROS) have been implicated in O_2 sensing in the three cell types mentioned (Acker & Xue, 1995; Wolin *et al.* 1999; Zhu & Bunn, 1999), under the generic name of redox models of O_2 sensing and transduction. According to one redox model (Acker & Xue, 1995), hypoxia would decrease the activity of a phagocyte-like NAD(P)H oxidase with subsequent decrease in ROS production. The falling levels of ROS, or the concomitant increase in the reduced to oxidized glutathione ratio (GSH/GSSG), would cause a reduction of the disulfide bonds in the proteins forming the K^+ channels, thereby leading to a decrease in their opening probability, followed by cell depolarization, activation of voltage-dependent Ca^{2+} channels and release of neurotransmitters from the chemoreceptor cells (López-

Barneo *et al.* 1988; Post *et al.* 1992; see Gonzalez *et al.* 1994). Although the involvement of NAD(P)H in O_2 sensing in the three cell types (but not in neuroepithelial bodies; Fu *et al.* 2000; O'Kelly *et al.* 2000) has been questioned (Gleadle *et al.* 1995; Archer *et al.* 1999; Obeso *et al.* 1999; Dvorakova *et al.* 2000; Roy *et al.* 2000), the decrease in ROS production and the increase in GSH/GSSG continues to be considered a critical step in low P_{O_2} sensing: the most recent hypothesis is that hypoxia decreases the production of ROS in mitochondria (Archer *et al.* 1999; Lahiri & Acker, 1999). Opposing these notions, Chandel and co-workers (1998) have observed that the mitochondrial rate of production and levels of ROS in the cells increase during hypoxia. They have proposed that increased ROS levels could mediate the hypoxic activation of CB chemoreceptor cells (Chandel & Schumacker, 2000).

The aim of this work was to determine the GSH/GSSG ratio in the CB and to monitor the response of chemoreceptor cells in normoxia, hypoxia and during the experimental modification of the GSH/GSSG ratios produced by incubation of the CBs with *N*-acetylcysteine (NAC). NAC is a precursor of GSH, increasing the GSH/GSSG ratio, and a scavenger of several ROS molecules (Dent *et al.* 1997; Halliwell & Gutteridge, 1999). Thus, according to the redox model in which hypoxia decreases ROS levels, NAC should mimic the natural hypoxic stimulus and activate chemoreceptor cells. On the other hand, according to the redox model in which hypoxia increases ROS levels, NAC should inhibit chemoreceptor cells and oppose hypoxic stimulation. To monitor the activation of chemoreceptor cells we have measured their release of ^{3}H-CA in CBs whose CA deposits have been labelled by prior incubation with the natural precursor ^{3}H-tyrosine. We found that hypoxia did not significantly alter the levels or the ratio GSH/GSSG. On the contrary, NAC increased the level and the GSH/GSSG ratio, but did not alter the release of ^{3}H-CA induced by hypoxia. We conclude that the GSH/GSSG ratio or ROS levels do not mediate O_2 sensing.

METHODS

Surgery and CB isolation and identification

Adult New Zealand White rabbits (2–2.5 kg) were anaesthetized with 40 mg kg^{-1} sodium pentobarbital (Sigma, Madrid, Spain) dissolved in saline and administered through the lateral vein of the ear. The rabbits were tracheostomized and blocks of tissue containing the carotid bifurcations were removed and placed in a lucite chamber containing ice-cold Tyrode solution (mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; glucose, 5.5; Hepes, 10) adjusted to pH 7.40 with 1 N NaOH. The CBs (6–12 per experiment) were cleaned of surrounding connective tissue and collected in fresh Tyrode solution. Animals were killed with an intracardiac overdose of pentobarbital (100–200 mg). All measures were taken to ensure the animals did not suffer distress at any time. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Valladolid.

Calf CBs were obtained from the slaughterhouse. Three to five minutes after the death of the animals the carotid bifurcations were removed and stored in ice-cold Tyrode solution. The bifurcations were placed in a lucite chamber and the CBs, formed by one or more lobules, were cleaned. The CBs were identified by their proximity to some of the arterial branches of the carotid sinus, by their pink-reddish appearance and by their penetration by thin nerve filaments. The identity of the calf CB was confirmed by histological examination of cryostat sections obtained from CB lobules fixed in 0.4% paraformaldehyde, by immunocytochemical staining of tyrosine hydroxylase in the same type of sections (Fig. 1) and by HPLC analyses of the CAs (Vicario *et al.* 2000) in homogenates of 'small pieces' of the CB. Levels of endogenous dopamine (597.9 ± 120.8 pmol (mg tissue)$^{-1}$) and of noradrenaline (708.0 ± 123.3 pmol (mg tissue)$^{-1}$), as well as dopamine/noradrenaline ratios (0.86 ± 0.09) found in 12 samples of calf carotid bodies, are comparable to those previously reported for the cat CB (see Gonzalez *et al.* 1994).

For release experiments (see following section), the calf CBs (10–20 mg) were cut into 'small pieces' of 0.5–2 mg and each piece was treated as an individual rabbit CB. To measure GSH and GSSG, five CBs were pooled to obtain samples of 50 mg or more.

Labelling of the catecholamine (CA) deposits and release of labelled CA

The six to 12 rabbit CBs or calf CB pieces used per experiment were incubated (2 h; 37 °C) in Tyrode solution containing 100 μM 6-methyl-tetrahydropterine (a tyrosine hydroxylase cofactor; Sigma, Madrid), 1 mM ascorbic acid (a cofactor of dopamine-β-hydroxylase) and 40 μM of the catecholamine natural precursor ^{3}H-tyrosine (20 Ci mmol^{-1}; Amersham Ibérica, Madrid). Each rabbit CB (~0.4 mg) synthesized ~12 pmol of ^{3}H-dopamine (^{3}H-DA; ~3×10^5 d.p.m.) and ~1 pmol of ^{3}H-noradrenaline (^{3}H-NA; ~2.5×10^4 d.p.m.) (Obeso *et al.* 1992). Twelve pieces of calf CB (from four different animals) analysed by HPLC (Vicario *et al.* 2000) contained 28.6 ± 4.0 pmol (mg tissue)$^{-1}$ of ^{3}H-DA and 0.56 ± 0.06 pmol (mg tissue)$^{-1}$ of ^{3}H-NA.

At the end of the labelling period, CBs were transferred to new vials (one rabbit CB or a piece of calf CB per vial) containing 4 ml of precursor-free Tyrode bicarbonate solution (24 mM NaCl was replaced with 24 mM $NaHCO_3$). Incubating solutions were continuously bubbled with 21% O_2–5% CO_2 (balanced with nitrogen) saturated with water vapour. During a period of 2 h the solution was renewed every 30 min and discarded; in this washing period most of the precursor and the labile pool of ^{3}H-CA are lost; afterwards the basal release of ^{3}H-CA decays monotonically (Obeso *et al.* 1999). After this washing period the release experiment was started and the incubating solutions were renewed every 10 min and collected for analysis of their ^{3}H-CA content. To study the effects of NAC on the basal release of ^{3}H-CA, the CBs were incubated with normoxic solutions (equilibrated with 21% O_2–5% CO_2) for 90 min, and during 50 min (from minutes 20 to 70) the solutions contained NAC at concentrations from 20 μM to 10 mM. To study the effects of NAC on the stimulus-evoked release of ^{3}H-CA the CBs were stimulated twice (from minutes 20 to 30 and from minutes 100 to 110) by incubating them with hypoxic (7% O_2) or high K^+ (35 mM)-containing solutions; NAC was applied prior to and during the second stimulus application (minutes 60 to 110; see Fig. 5). The stimulus-evoked release of ^{3}H-CA was calculated as the d.p.m. above basal normoxic release and expressed as a percentage of the tissue content prior to the application of the stimulus. The ^{3}H-CA tissue content at a given moment was calculated by adding together the ^{3}H-CA present in the tissue at the end of the experiment and the ^{3}H-CA present in the solutions collected between the moment of interest and the end of the experiment. The effect of NAC on the stimulus-evoked release of ^{3}H-CA was assessed by comparing the ratios of the evoked release in the second presentation

of the stimulus to that of the first (S_2/S_1) in NAC-treated CBs, with those ratios obtained in drug-free CBs. In an additional group of experiments, the effects of NAC on the release of ^{3}H-CA elicited by the Ca^{2+} ionophore ionomycin were studied. In these experiments, the ionophore was applied only once (from minutes 60 to 70) due to the difficulty of washing it out, and the amounts of ^{3}H-CA released in control and NAC-treated (from minutes 20 to 70) CBs were compared. At the end of the experiment, the CBs were immersed in 0.4 N perchloric acid, glass-to-glass homogenized at 0 °C, centrifuged for 10 min in a Beckman microfuge in a cold room. The supernatant and the incubation media were processed for analysis of ^{3}H-CA. The analysis included the following: acidification of the collected incubating solutions to pH 3.2 with a mixture of glacial acetic and ascorbic acid to avoid degradation of CA; bulk adsorption into alumina of all released catechols at a pH of 8.6 – achieved by addition of 2.5 M Tris buffer at pH 8.6; and intense washing of alumina columns with distilled water and bulk elution of all catechols with 1 ml 1 N HCl. The alumina eluates were counted in a liquid scintillation spectrometer and the released ^{3}H-CA expressed as d.p.m. per 10 min (Fidone *et al.* 1982; Obeso *et al.* 1992).

Measurement of GSH and GSSG

Three to five calf CBs/samples were cut into small pieces and incubated for 40 min at 37 °C in 20 % O_2–5 % CO_2-equilibrated Tyrode bicarbonate solution to allow the recovery of tissue after the surgical manipulations (Obeso *et al.* 1985). In control CBs, the incubating solution was renewed at minutes 40, and the incubation was maintained for a further 10 min. In hypoxic CBs this last incubation of 10 min was made in a solution equilibrated with 5 % O_2–5 % CO_2. NAC-treated samples were incubated as controls, but solutions contained 2 mM NAC. After incubation, tissues were placed in Eppendorf tubes containing 100 μl of 5 % 5-sulfosalicylic acid (SSA; Sigma) and 0.25 mM ethylenediaminetetraacetic acid (EDTA) to prevent oxidation of GSH and to inhibit GSH-utilizing enzymes. After 10 min in SSA, the tissues were weighed and the volume of SSA solution was brought to 5 × tissue weight. Tissues were glass-to-glass homogenized at 0 °C, centrifuged (10 min; 4 °C) in a microfuge and the supernatant used to measure GSH and GSSG.

Measurements were made using Griffith's (1980) method (see Punchard & Kelly, 1996). GSH reacts non-enzymatically with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to generate GSSG and the highly coloured 5-thio-2-nitrobenzoic acid (TNB; peak absorbance at 420 nm); the GSSG formed is back-reduced to GSH by glutathione reductase coupled to NADPH oxidation. In this cycling assay, concentrations of the reactants are chosen so that the rate of colour formation is linear with time, with the slope of the line (Δabsorbance/Δtime (min); $\Delta A/\Delta t$ (min)) being directly proportional to the concentration of total glutathione (GSH + GSSG; GSt). This allows the computer-assisted on-line construction of standard curves relating GSt concentrations to the slopes of their assays. Since this relationship is linear, it is possible to measure the concentration of GSt in identically treated test samples by interpolation. GSSG was similarly determined except that (1) GSH is masked by derivatization with 2-vinylpyridine, and (2) the size of the sample is higher owing to the much lower concentration of GSSG than of GSH. Standards and tissue homogenates were assayed in triplicate. Assay mixtures contained: 700 μl of daily-prepared 0.3 mM NADPH solution, 100 μl of 6 mM DTNB solution stored at −20 °C, 200 μl of water minus the sample volume (1–5 μl of supernatant for GSt and up to 20 μl for GSSG; 1 μl

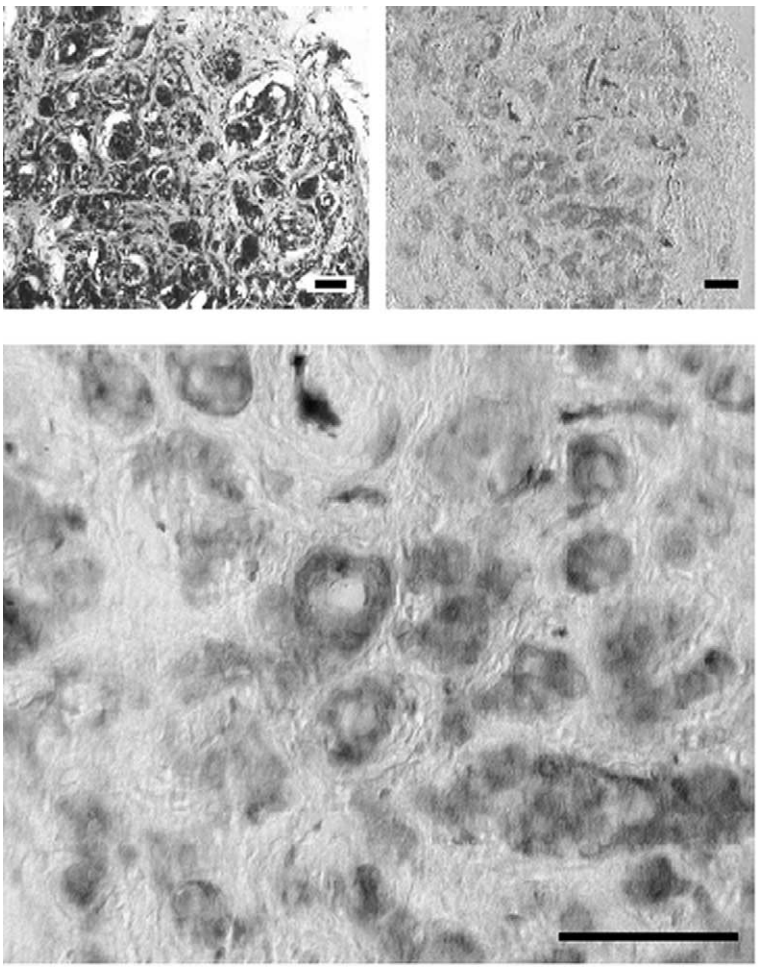

Figure 1. Histological identification of the calf carotid body

Top left shows a low magnification cryostat section stained with cresyl violet evidencing the typical clusters of glomic tissue in the vicinity of capillaries. Top right shows a cryostat section immunostained for tyrosine hydroxylase and developed with diaminobenzidine after incubation with a secondary antibody conjugated with peroxidase. Note the cluster-like distribution of the carotid body tissue positive to tyrosine hydroxylase. Bottom shows a detail of the previous section at higher magnification. Calibration bars are 50 μm.

is equivalent to 0.2 mg tissue; blanks contained a volume identical to the sample volume of 4.31 % SSA) and glutathione reductase (Sigma, cat. no. G3664; 1 and 5 units for GSt and GSSG assays, respectively). All reactants were dissolved in a phosphate buffer (0.125 mM; pH 7.5) containing EDTA at 6.3 mM. Measurements of glutathione were not possible in the rabbit CB. Its small size (~400 μg per CB) would require at least six animals per single unduplicated value.

Statistics

Significance of the differences observed between groups was assessed by the use of Student's two-tail t test for unpaired data. Means ± S.E.M. are given.

RESULTS

Validation of the glutathione assay: glutathione levels in the calf CB

The lack of data on the levels of glutathione in the CB and the need for using high concentrations of NAC, which might react with DTNB, the colour generating reactant of the assay (Punchard & Kelly, 1996), suggested that the assay should be validated by intraexperimentally measuring glutathione in the CB and in tissues with known glutathione levels. It was also necessary to construct standard curves with concentrations of NAC similar to those used in our experiments. Rat liver GSt and GSSG contents were 5.91 ± 0.78 and 0.11 ± 0.03 nmol (mg fresh tissue)$^{-1}$, respectively, ($n = 7$) which are within the range of most published works (Fig. 2*A*; Halliwell & Gutteridge, 1999). In the calf CBs, the levels of GSt and GSSG assayed in parallel were lower, 0.82 ± 0.10 ($n = 6$) and 0.025 ± 0.003 nmol (mg fresh tissue)$^{-1}$ ($n = 6$), respectively (Fig. 2*B*). The ratios GSH/GSt for rat liver and calf CB were 0.98 ± 0.007 and 0.95 ± 0.014, respectively (Fig. 2*C*). The GSH/GSSG ratio, which is an alternative way of expressing the relative proportions of GSH and GSSG, was over 50 for the rat liver and over 30 for the CB. Standard curves made for GSH and GSSG dissolved in control solutions (phosphate buffer–EDTA–SSA) and in solutions containing 2 mM NAC were almost indistinguishable (Fig. 2*D* and *E*).

Effect of hypoxia and NAC treatment on the CB glutathione levels

The glutathione contents measured in 12 normoxic (incubated at $P_{O_2} \approx 133$ mmHg), 10 hypoxic ($P_{O_2} \approx 33$ mmHg) and six NAC-treated ($P_{O_2} \approx 133$ mmHg; 2 mM NAC)

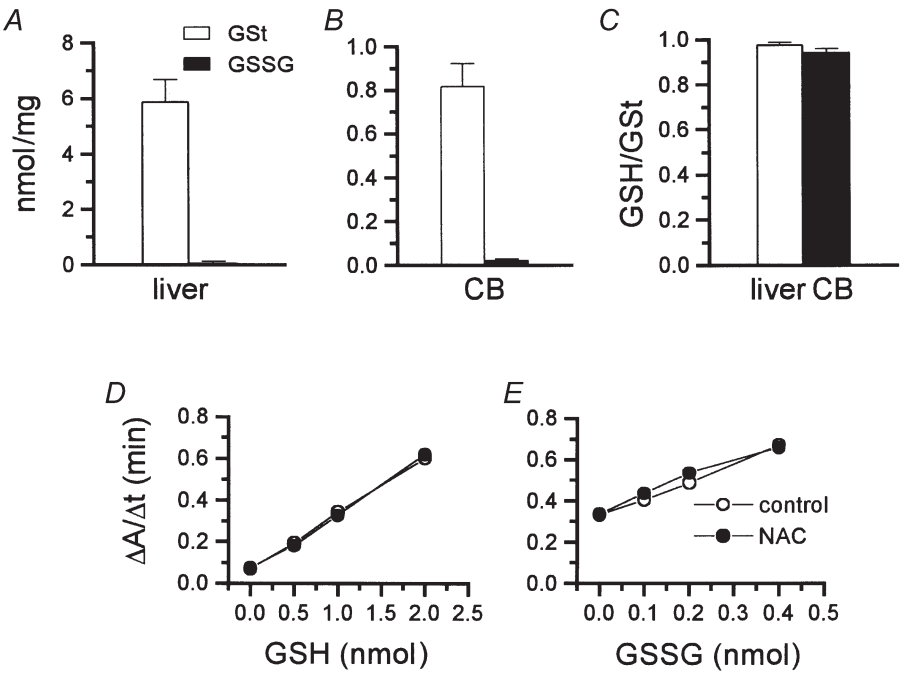

Figure 2. Validation of the glutathione assay conditions

A and *B*, total (GSt) and oxidized glutathione (GSSG) levels in the rat liver and calf carotid body (CB), respectively. *C*, reduced to total glutathione (GSH/GSt) ratios in the rat liver and calf carotid body. *D* and *E*, standard curves for reduced (*D*) and oxidized (*E*) glutathione in control conditions and in the presence of NAC. Least-squares linear fitting for the GSt curves were: $y = 0.27x + 0.07$ for control and $y = 0.27x + 0.06$ for NAC-containing samples ($r^2 > 0.99$ in both cases). For GSSG curves the equations were: $y = 0.85x + 0.32$ ($r^2 > 0.99$) for control and $y = 0.80x + 0.35$ ($r^2 > 0.98$) for NAC-containing samples. Assay mixtures consisted of 700 μl of a 0.3 mM NADPH solution prepared daily, 100 μl of a 6 mM DNTB solution stored at −20 °C in aliquots to be used in a given day, 200 μl of water minus sample volume (1–5 μl of supernatant, equivalent to 0.2–1.0 mg tissue, for GSt and up to 20 μl, equivalent to up to 4 mg tissue, for GSSG; blanks contained a volume identical to the sample volume of 4.31 % SSA) and glutathione reductase (1 unit and 5 units for GSt and GSSG assays, respectively). All reactants were dissolved in a phosphate buffer (0.125 mM; pH 7.5) containing EDTA at 6.3 mM.

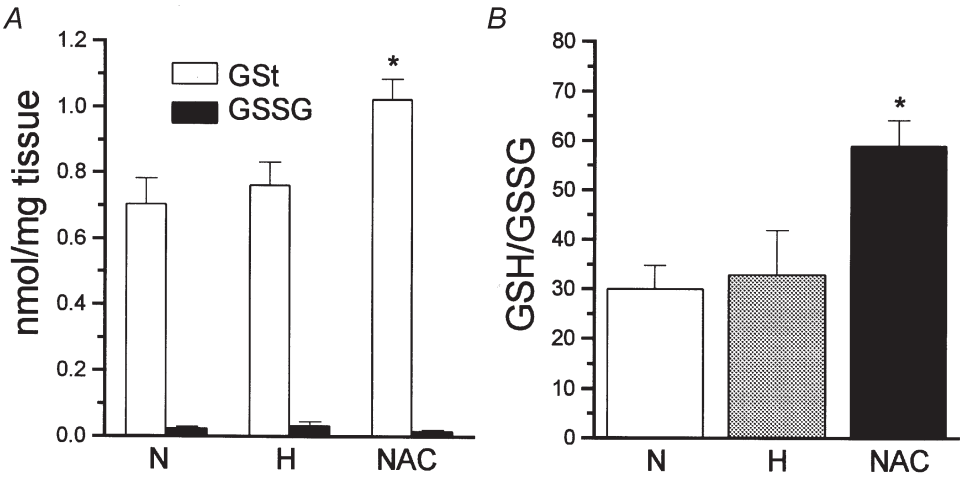

Figure 3. Effects of hypoxia and *N*-acetylcysteine (NAC) on glutathione levels in the calf carotid body

A, levels of total (GSt) and oxidized glutathione (GSSG) in normoxic (N), hypoxic (H) and NAC-treated (NAC) CBs. *B*, reduced to oxidized glutathione ratios in normoxic, hypoxic and NAC-treated CBs. Data are means ± S.E.M. and $n = 12$ (normoxic), $n = 10$ (hypoxic) and $n = 6$ (NAC-treated). *$P < 0.02$.

CB tissue samples are shown in Fig. 3. Absolute levels of GSt in normoxic tissues were 0.71 ± 0.07 nmol (mg tissue)$^{-1}$, in hypoxic tissues 0.76 ± 0.07 nmol (mg tissue)$^{-1}$ and in NAC-treated tissues 1.03 ± 0.06 nmol (mg tissue)$^{-1}$ ($P < 0.02$ *vs.* previous groups); GSSG levels in normoxic, hypoxic and NAC-treated tissues amounted, respectively, to 0.026 ± 0.003, 0.034 ± 0.009 and 0.019 ± 0.002 nmol (mg tissue)$^{-1}$ (Fig. 3*A*). The GSH/GSSG ratios in the groups are shown in Fig. 3*B*. They were 30.14 ± 4.67 in the normoxic group, 33.03 ± 6.88 in the hypoxic group and 59.05 ± 5.05 in the NAC-treated group ($P < 0.001$ and $P < 0.02$ *vs.* the normoxic and hypoxic group, respectively). The data indicate that hypoxia of moderately high intensity did not significantly alter GSH/GSSG levels or ratios in the CB while NAC treatment significantly increased GSH levels and GSH/GSSG ratios.

Effects of NAC on normoxic and hypoxic release of ^{3}H-CA from the calf and rabbit CB

According to one redox model of O_2 sensing (see Introduction), the increase in the GSH/GSSG ratio (and the decrease in ROS levels, see Dent *et al.* 1997; Villagrasa *et al.* 1997; Halliwell & Gutteridge, 1999) produced by

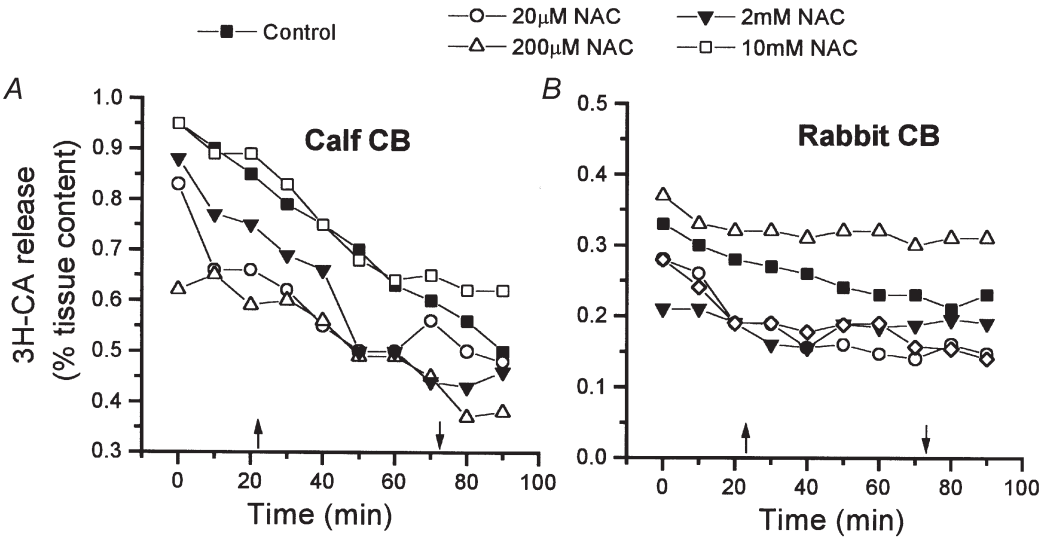

Figure 4. Effect of several concentrations of *N*-acetylcysteine (NAC) on the basal release of ^{3}H-CA in the calf (*A*) and the rabbit CB (*B*)

N-Acetylcysteine at the concentrations shown was present in the incubating solution for 50 min (between arrows). Data are means of 10–12 individual values for the calf and 5–8 individual values for the rabbit. Standard error (<15%) bars have been eliminated for clarity.

NAC treatment should trigger a neurosecretory response in normoxia and potentiate the secretory response induced by a moderate hypoxic stimulus. According to the other redox model it should produce the opposite effect. Therefore, we tested for the capacity of NAC to alter the release of ^{3}H-CA from the CB of calf, a species in which we have measured GSH levels, and from the CB of rabbit, a species whose release of ^{3}H-CA is well characterized (Fidone *et al.* 1982; Obeso *et al.* 1992, 1999). The time courses (50 min) of the effects of several concentrations of NAC (20 μM to 10 mM) on the normoxic release of ^{3}H-CA in the calf and rabbit CB are shown in Fig. 4*A* and *B*, respectively. None of the concentrations altered the ongoing normoxic release of ^{3}H-CA in the CB of either species.

The effect of NAC on the release of ^{3}H-CA elicited by hypoxia ($P_{O_2} \approx 46$ mmHg; 10 min) was studied in pairs of CBs. The CBs were stimulated twice in the absence (control) or the presence (experimental) of 2 mM NAC applied during the 40 min prior to and during the 10 min of the second hypoxic stimulus (Fig. 5*A* and *B*, calf CB; Fig. 5*D* and *E*, rabbit CB). The ratios of the evoked release in the second/first stimulus (S_2/S_1) for control and experimental CBs in this particular experiment were calculated and plotted in Fig. 5*C* (calf) and Fig. 5*F* (rabbit). Figure 6 shows the mean S_2/S_1 obtained in control and NAC-treated calf CB pieces and intact rabbit organs. In the calf CB tissues, the S_2/S_1 ratio in control organs was 0.73 ± 0.12 ($n = 10$) and in NAC-treated tissues it was 0.71 ± 0.12 ($n = 10$). In the rabbit CB, the S_2/S_1 ratios in control and NAC-treated CBs were, respectively, 0.52 ± 0.07 and 0.61 ± 0.10 ($n = 12$). The observed differences were not statistically significant. In an additional experiment with calf CB tissue, NAC was applied for only 10 min prior to and during the 10 min of hypoxic stimulation. The S_2/S_1 ratios obtained in this experiment were not statistically different in control (0.83 ± 0.14; $n = 6$) and NAC-treated CB pieces (0.73 ± 0.08; $n = 6$) (data not shown). In all these experiments (see Fig. 5*B* and *E*), the lack of effect of 2 mM NAC on the basal release of ^{3}H-CA was confirmed.

Effects of NAC on the release of ^{3}H-CA induced by high external K^+ and ionomycin in the calf and rabbit CB

In permeabilized chromaffin cells it was demonstrated that sulfhydryl reagents blocked Ca^{2+}-induced CA secretion, suggesting that thiol groups of proteins involved in the exocytotic machinery were critical in

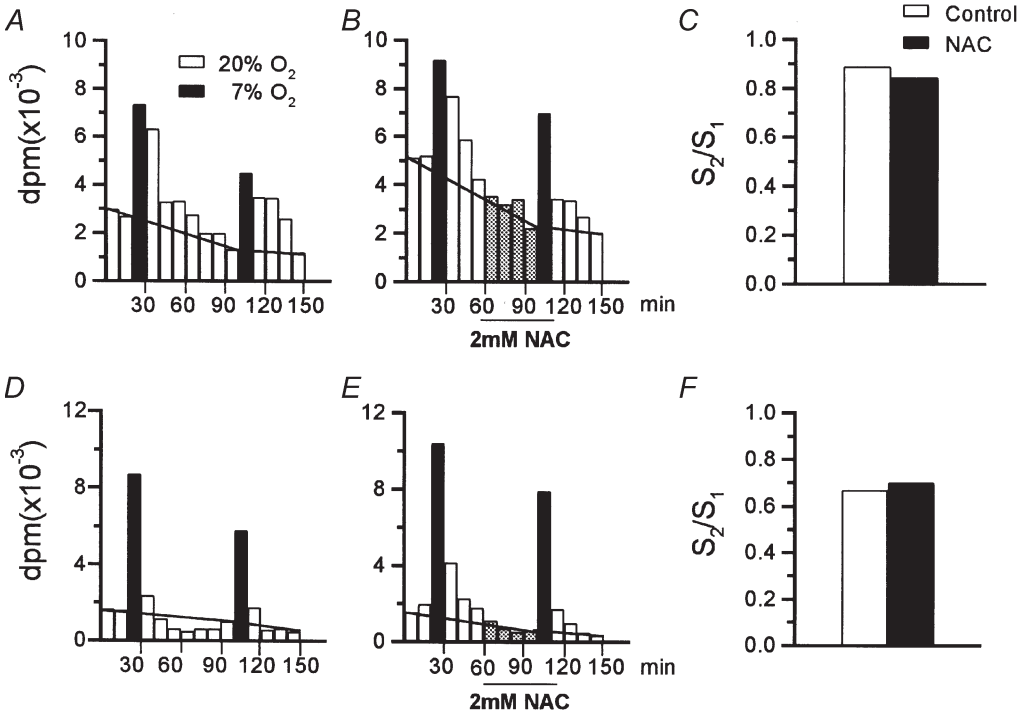

Figure 5. General protocol to study the effects of *N*-acetylcysteine (NAC) on the release of ^{3}H-CA induced by hypoxia in the calf and rabbit CB

A–C refer to the calf CB and depict a typical experiment with a pair of CBs showing the protocol used to test the effect of NAC (2 mM) on the release of ^{3}H-CA elicited by hypoxic stimulation (10 min incubation in a solution equilibrated with 7 % O_2–5 % CO_2 balanced with N_2, filled bars). The horizontal lines crossing the histograms in *A* and *B* of the figure separate the basal release (below) from the stimulus-evoked release (above the lines). *C*, the ratio of the low P_{O_2}-evoked release (d.p.m.) in the second presentation of the stimulus (S_2) to that in the first presentation (S_1). *D–F* show an identical experiment carried out with a pair of rabbit CBs.

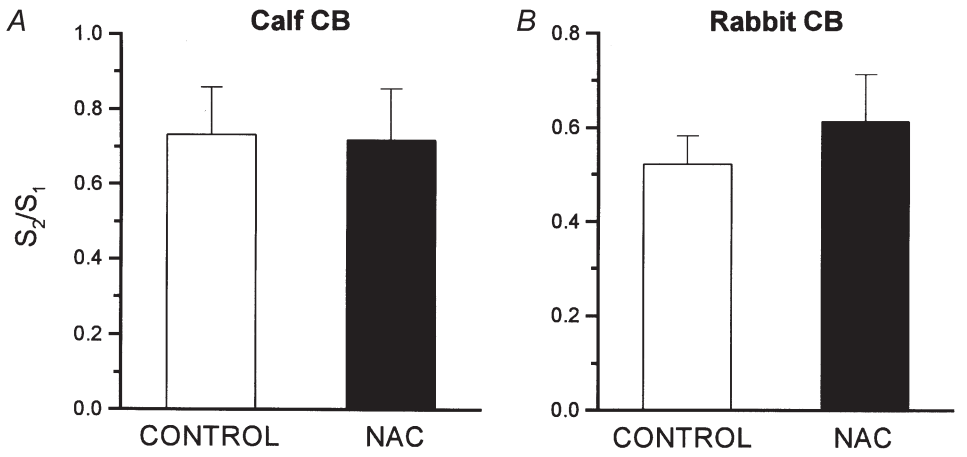

Figure 6. Effects of *N*-acetylcysteine (NAC) on the neurosecretory response elicited by hypoxia in chemoreceptor cells of the CB

The figure shows means ± S.E.M. of S_2/S_1 ratios obtained in 10 experiments like that shown in Fig. 5 for the calf CB (*A*) and 12 experiments with rabbit CBs (*B*).

controlling the release process (Augustine *et al.* 1987). Recently, two *N*-ethylmaleimide-sensitive factors controlling neurotransmitter release have been identified at the molecular level (Schweizer *et al.* 1998). In addition, Ca^{2+} channels, which are known to participate in the release of CA induced by hypoxia (Obeso *et al.* 1992), are also regulated by redox agents (Fearon *et al.* 1999). Therefore, the possibility exists that the effects of the altered GSH/GSSG quotients produced by NAC at the O_2-sensing machinery are masked by modifications of thiol groups and disulfide bonds in cell proteins located downstream in the transduction cascade (i.e. Ca^{2+} channels and/or exocytotic machinery). To test this possibility, we have studied the effects of NAC on the release of ^{3}H-CA induced by high extracellular K^+ and by ionomycin. The release response induced by high K^+ presumably involves all the processes of the hypoxic response except the O_2-sensing step (Gonzalez *et al.* 1994), and the response elicited by ionomycin bypasses all the steps of the hypoxic release except exocytosis itself (Obeso *et al.* 1992).

The protocols for the experiments with high extracellular K^+ (35 mM) were identical to those used for hypoxia. The S_2/S_1 ratios for control and NAC-treated calf CB tissues were 0.54 ± 0.06 and 0.51 ± 0.04, respectively ($n = 10$; Fig. 7*A*); in the rabbit CBs the ratios were 0.64 ± 0.07 ($n = 7$) for control and 0.77 ± 0.08 ($n = 7$) for NAC-treated tissues (Fig. 7*B*). In neither species was the difference statistically significant. In the experiments

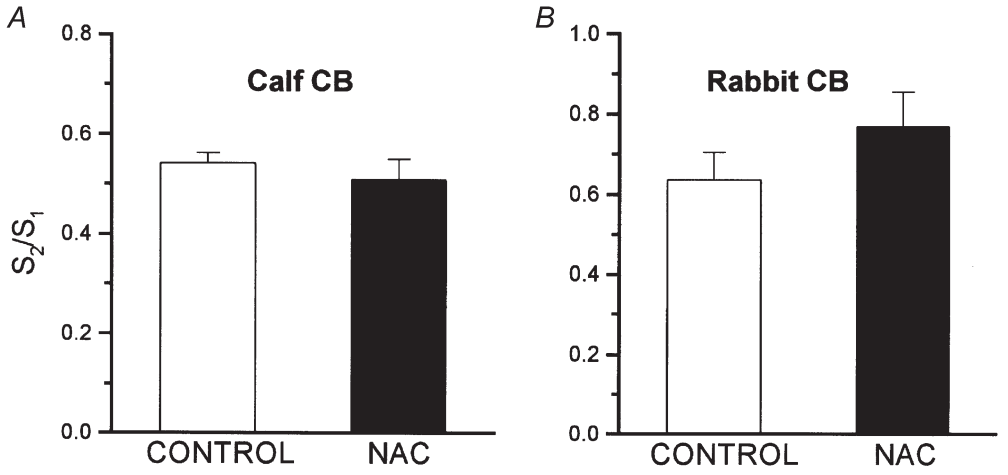

Figure 7. Effects of *N*-acetylcysteine (NAC) on the neurosecretory response elicited by high external K^+ in chemoreceptor cells of the CB

Experimental protocol was as given in Fig. 5. S_2/S_1 ratios in control and NAC-treated calf (*A*) and rabbit (*B*) CBs refer to two sequential stimulations with high external K^+ (incubation with 35 mM K^+ for 10 min). Data are means ± S.E.M. of 10 individual values for the calf CBs and 7 individual values for the rabbit CBs. The differences between control and NAC-treated organs are not statistically significant.

with ionomycin, the ionophore (20 μM) was applied only once to control and to NAC-treated CB tissues. Figure 8*A* and *B* shows a single experiment carried out in a pair of rabbit CBs. Note the absence of effect of NAC on the basal release and the similarity of the magnitude and time course of the release induced by ionomycin (d.p.m. above dotted horizontal lines in the figures) in the absence and in the presence of NAC. The ^{3}H-CA released by ionomycin in the calf CBs represented 18.9 $\pm$ 1.8 % of the tissue content in control organs and 17.26 $\pm$ 1.12 % in NAC-treated tissues ($n = 10$; $P > 0.05$; Fig. 8*C*). In the rabbit CBs ionomycin released 10.94 $\pm$ 1.71 % and 10.77 $\pm$ 1.39 % of the ^{3}H-CA content in control and NAC-treated tissues, respectively ($n = 10$; $P > 0.05$; Fig. 8*D*).

DISCUSSION

The aim of the present work has been to test some of the premises of the redox models of O_2 chemoreception. Specifically, we have measured the GSH/GSSG ratio in normoxic and hypoxic CBs to verify if hypoxia alters this quotient in a given direction. Additionally, we have experimentally increased the GSH/GSSG ratio by treatment of the CBs with NAC to determine the modifications of the neurosecretory response (release of ^{3}H-CA) of chemoreceptor cells produced by this experimental manoeuvre. The results indicate that hypoxic stimulation of CBs at moderately high intensity ($P_{O_2} \approx 33$ mmHg) does not alter the GSH/GSSG quotient. The increase in the GSH/GSSG quotient produced by NAC does not alter the normoxic release of ^{3}H-CA nor the release response elicited by hypoxia, high external K^+ and the Ca^{2+} ionophore ionomycin.

At the outset of the discussion, it might be of interest to clarify some aspects of the metabolism of ^{3}H-CAs, including the significance of their secretion during CB stimulation and their putative physiological role in chemoreception. Chemoreceptor cells detect P_{O_2} and respond to graded hypoxia with a proportional increase in the release of CA (mostly DA) (see Gonzalez *et al.* (1994) for data in the rabbit and cat, and Vicario *et al.* (2000) for data in the rat). Therefore, even if the significance of CA in CB function is in dispute (Gonzalez *et al.* 1997 *vs.* Zapata, 1997), the release of CA represents a direct measure of the output of the hypoxic chemoreception in chemoreceptor cells. However, it should also be mentioned that on repetitive stimulation of the CB (Donnelly, 1995), the release of ^{3}H-CA tends to decrease while the postsynaptic response decreases with a slower time course, as is the case in many other catecholaminergic structures (for references, see Gonzalez *et al.* 1997).

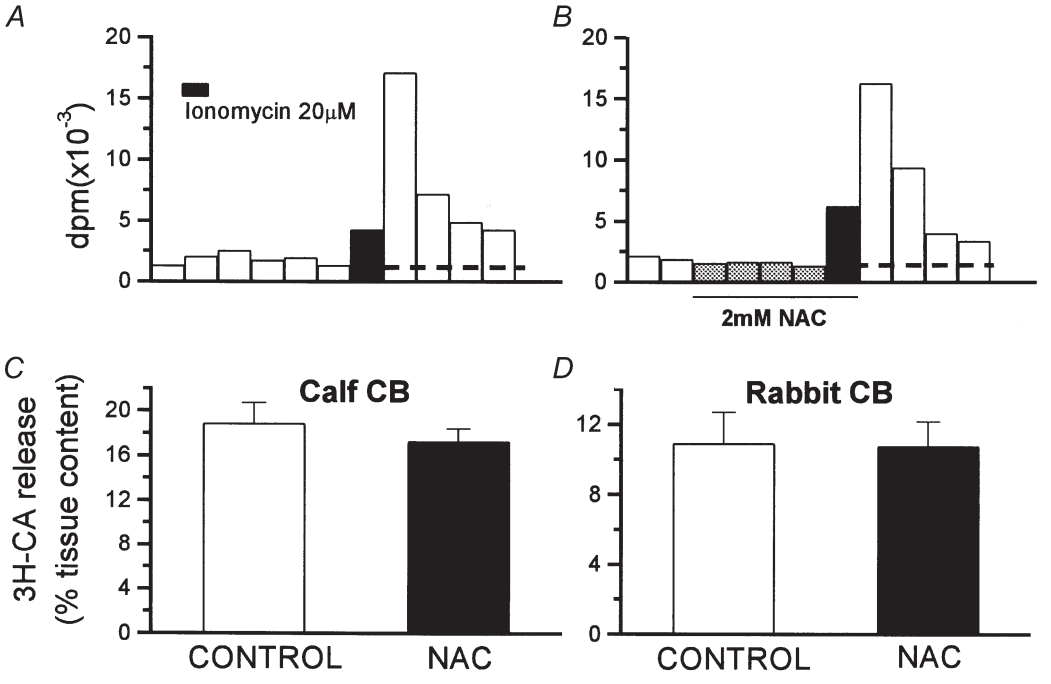

Figure 8. Effects of *N*-acetylcysteine (NAC) on the release of ^{3}H-CA elicited by ionomycin in the rabbit and calf CBs

A and *B*, a single experiment with a pair of rabbit CBs to illustrate the protocol followed in studying the effects of NAC on the release of ^{3}H-CA induced by ionomycin. The horizontal lines crossing the bars separate the basal (below the line) from the ionomycin-evoked (above the line) release of ^{3}H-CA. *C* and *D*, means of the release evoked by ionomycin (expressed as a percentage of tissue content) in the control and NAC-treated calf (*C*; $n = 10$) and rabbit CBs (*D*; $n = 10$). The differences observed were not statistically significant.

In the case of the calf CB, the data available on the metabolism of CA are scarce. Bader *et al.* (1981) using fluorometric methods found that the calf CB is, like the cat CB, a mixed dopaminergic and noradrenergic organ with levels of DA and NA of ~500 and 600 nmol (g fresh tissue)$^{-1}$, respectively. The data of the present study (endogenous DA and NA levels of 597.9 ± 120.8 and 708.0 ± 123.3 pmol (mg tissue)$^{-1}$, respectively, $n = 12$; synthesis rates of 28.6 ± 4.0 pmol (mg tissue)$^{-1}$ (2 h)$^{-1}$ for ^{3}H-DA and 0.56 ± 0.06 pmol (mg tissue)$^{-1}$ (2 h)$^{-1}$ for ^{3}H-NA), confirm the mixed noradrenergic–dopaminergic nature of the calf CB with NA levels slightly higher than DA levels, as is the case in the cat CB (Gonzalez *et al.* 1994). These data indicate that in the calf, as in other species, the turnover rate for DA is much higher than for NA (Gonzalez *et al.* 1994; Vicario *et al.* 2000). Therefore, the findings would suggest that in all species studied DA should be more significant than NA in chemoreceptor signalling at the chemoreceptor cell–sensory nerve ending synapse. The release of ^{3}H-CA induced by moderate hypoxia, high external K^+, ionomycin (Figs 5–8) and intense hypoxia (2 % O_2; not shown) in the calf CB compares favourably with the release observed in the rabbit CB.

The absolute levels of GSt and the GSH/GSt ratios of almost 1.0, which we found in the liver, are comparable to those found by other authors (Halliwell & Gutteridge, 1999). In the CB, the GSH/GSt ratio is comparable, but the absolute levels of GSt are lower than the levels found in the rat liver, and more like those reported for rat brain (Halliwell & Gutteridge, 1999). The assay was linear for both forms of glutathione in the range of concentrations found in the CB, and the linearity was not affected by the presence of NAC. Although NAC contains a sulfhydryl group that might react with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) – the colour-generating reagent in the assay mixture – the enzymatic cycling mediated by glutathione reductase provides the required assay specificity to measure glutathione, and prevents the interference by other compounds having the sulfhydryl group (Punchard & Kelly, 1996). Therefore, the levels of glutathione measured in the CB correspond to genuine GSt and GSSG.

The first specific aim of our work was to test for the effects of hypoxia on GSH/GSSG ratios in the CB. The strength of the hypoxic stimulus selected to measure glutathione was of moderately high intensity ($P_{O_2} \approx 33$ mmHg) in order to attain a good level of chemoreceptor cell activation without compromising cell functioning during the period of stimulation. As Fig. 3 shows, 10 min of stimulation with hypoxia of moderately high intensity did not significantly alter the levels or the GSH/GSSG ratio. This finding does not support the principal assumption of one of the redox models for oxygen sensing in chemoreceptor cells. This proposal is that hypoxia acts via an increase in GSH/GSSG ratio with subsequent reduction of disulfide bonds in K^+ channel proteins (Archer *et al.* 1993; Acker & Xue, 1995; Lahiri & Acker, 1999), which in turn decreases the opening probability of K^+ channels to produce chemoreceptor cell depolarization and release of neurotransmitters. Our findings would indicate that hypoxia activates chemoreceptor cells to promote neurotransmitter release without altering the GSH/GSSG quotient. However, the negative result, i.e. the lack of effect of hypoxia on glutathione level and GSH/GSSG quotient in the entire calf CB, could be a 'false' negative. Due to the heterogeneity of the CB, with chemoreceptor cells representing less than 50 % of the CB tissue (Verna, 1997), the possibility exists that hypoxia is in fact altering the GSH/GSSG ratio in chemoreceptor cells, as the redox models propose, but that this effect is undetectable with assay of the entire organ. If this were the case, our conclusions would be unjustified.

To circumvent this potential pitfall, we have used the strategy of increasing the GSH/GSSG quotient and of decreasing ROS levels (Dent *et al.* 1997; Villagrasa *et al.* 1997; Halliwell & Gutteridge, 1999) in all cell types of the CB by incubating the organs with NAC and by measuring the release of ^{3}H-CA as a specific parameter of the activation of chemoreceptor cells. If the redox models are correct, NAC should mimic hypoxia and evoke the release of ^{3}H-CA even in normoxic conditions and potentiate the release response evoked by submaximal hypoxic stimuli (or it should inhibit the release, according to the alternative redox model; see Introduction). As expected (Dent *et al.* 1997; Villagrasa *et al.* 1997; Halliwell & Gutteridge, 1999), incubation of the calf CB with 2 mM NAC for 50 min was effective in increasing the levels of GSt (Fig. 3*A*) with almost no change in GSSG (Fig. 3*A*), and therefore increasing the GSH/GSSG quotient (Fig. 3*B*). It was at the same time ineffective for altering the basal normoxic release of ^{3}H-CA in the calf and in the rabbit CB (Fig. 4). Thus, it seems that the increase in the GSH/GSSG quotient *per se* is not a sufficient requirement for activation of the neurosecretory response of chemoreceptor cells, as occurs with hypoxia. Additionally, the treatment with NAC was also ineffective for augmenting the release response elicited by a moderate hypoxic stimulus (Figs 5 and 6). Once again, these findings argue against the redox models of oxygen chemoreception. The argument is reinforced by the demonstration that NAC treatment does not alter downstream steps in the neurosecretory response, because the release of ^{3}H-CA elicited by high external K^+ and ionomycin (Figs 7 and 8) was not different in control and NAC-treated CBs.

Taken together, our findings do not support the redox models of O_2 sensing. These models, which envision ROS and GSH/GSSG ratios as playing a critical role in the process, occupy a mainstream position amongst current theories of O_2 sensing in the CB and pulmonary vessels. The redox hypothesis, or redox model, was first proposed by Archer *et al.* (1986) as the mechanism triggering hypoxic pulmonary vasoconstriction. They postulated

that O_2, through the generation of ROS during oxidative metabolism, controls the redox status of the cells as represented by the ratios GSH/GSSG and NADPH/NADP. Archer and co-workers (1986) reasoned that the rate of ROS production would parallel tissue P_{O_2} and therefore hypoxia would diminish the production of ROS. The decrease in ROS production would in turn lead to a decrease in the activity of glutathione peroxidase and to an increase in the GSH/GSSG ratio. The final step in the cascade leading to hypoxic vasoconstriction (as formulated at the time) was that a reduced status in the cells would activate Ca^{2+} influx and the contracting response. The description of O_2-sensitive K^+ currents in CB chemoreceptor cells (López-Barneo *et al.* 1988) was soon followed by the description of such currents in pulmonary artery smooth muscle cells (Post *et al.* 1992) and the ROS hypothesis in these cells changed to accommodate the new findings. Therefore, more recent proposals (Archer *et al.* 1993, 1999) postulate that a decrease of ROS and/or an increase in the GSH/GSSG quotient would lead to a decrease in the open probability of K^+ channels and cell depolarization, as a critical and initial step in the low P_{O_2} transduction cascade. With minimal modifications, Acker's group imported the redox model for O_2 sensing into the arterial CB chemoreception field (Acker & Xue, 1995; Lahiri & Acker, 1999). Redox models for O_2 sensing gained wide support from the observation that many types of K^+ channels change their gating properties when entire cells or isolated membrane patches are exposed to reducing or oxidizing agents, including dithiothreitol, GSH and H_2O_2 (e.g. Ruppersberg *et al.* 1991; Vega-Saez de Miera & Rudy, 1992; López-Barneo *et al.* 1999), albeit not always in the direction adequate for the hypothesis (Thuringer & Findlay, 1997; Liu *et al.* 1999; Lang *et al.* 2000). In addition, in a recent study from our laboratory (Perez-Garcia *et al.* 1999), we demonstrated that O_2 sensing is not equivalent to reduction of the sulfhydryl groups of channel proteins. Finally, several recent studies in different cell types indicate that hypoxia *increases* ROS levels (Marshall *et al.* 1996; Chandel *et al.* 1998; Hohler *et al.* 1999; see Chandel & Shumacker, 2000), implying that low P_{O_2} activates, *instead of inhibiting*, ROS production, and therefore the GSH/GSSG ratio decreases instead of increasing. However, the present study does not suggest that an increase in ROS or a decrease in GSH/GSSG is a significant signal in O_2 chemoreception in the CB, because NAC, which increases the GSH/GSSG ratio and decreases ROS levels (Dent *et al.* 1997; Villagrasa *et al.* 1997; Halliwell & Gutteridge, 1999), does not affect the hypoxic transduction cascade leading to the neurosecretory response. In this regard, we should stress the observations of Hohler *et al.* (1999), which show that the upregulation of tyrosine hydroxylase caused by hypoxia in PC12 cells (hypoxia also upregulates tyrosine hydroxylase in chemoreceptor cells, see Gonzalez *et al.* 1994 for references) is paralleled, but not induced, by increased ROS production during the hypoxic exposure. A lack of relationship between ROS production/levels, on the one hand, and chemoreceptor and pulmonary artery smooth muscle cell activation, on the other, is also inferred from the well-known observation that mitochondrial poisons, including rotenone, which decreases, and cyanide, which increases, ROS levels (Archer *et al.* 1993; Chandel & Schumacker, 2000), are both powerful chemostimulants (Zapata, 1997) and both produce pulmonary artery vasoconstriction (Archer *et al.* 1993).

Two final aspects of this study deserve consideration. The first relates to the meaning of the GSH/GSSG ratio, which we have measured as an index of the redox state of the cells. According to Archer *et al.* (1986), the ratios GSH/GSSG and NADPH/NADP represented the redox status of the cells in their formulation of the redox model, and indeed this is the case. However, since GSH/GSSG and NADPH/NADP pairs are intimately linked by glutathione reductase, which reduces GSSG to GSH using NADPH as cofactor, both quotients vary in parallel, and the measurement of one of them provides an adequate index of the redox status of the cells (Halliwell & Gutteridge, 1999). In fact, there exists an inter-relationship of the equilibria among the different redox pairs and ROS, such that the measurement of one of the elements (i.e. any ROS or any of the redox pairs) would provide an adequate index of the redox status of the cells. The second aspect relates to the possible origin of discrepancies regarding the effects of hypoxia in decreasing or increasing ROS levels and production. In Semenza's opinion, the discrepancies are most likely to be related to the demanding methodology used to directly measure these molecules (see Semenza, 2000). They do not seem to reflect cell specificity (Semenza, 2000) or different parameters of the hypoxic stimulus (e.g. duration or intensity), because Kietzmann *et al.* (1998), who found that hypoxia decreases the rate of ROS production, used hypoxic stimuli of similar duration and intensity to those used by Chandel *et al.* (1998) and found that hypoxia increases ROS production and levels.

In conclusion, the present study demonstrates that hypoxic stimuli of moderately high intensity do not affect the GSH/GSSG ratio in CB tissue. Incubation of CBs with NAC, an antioxidant and GSH precursor, augmented the GSH/GSSG ratios (and presumably reduced ROS levels), but did not activate or inhibit chemoreceptor cells, as judged by the lack of effect on the basal (normoxic) and hypoxia-induced release of ^{3}H-CA. Potential side effects of NAC on the exocytotic machinery, which might mask the effects of NAC on prior steps of the low P_{O_2} transduction cascade, were excluded by demonstrating that NAC is unable to alter the activation of chemoreceptor cells produced by high external K^+ and ionomycin. Our data do not support the redox models of O_2 sensing in the chemoreceptor cells of the CB.

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