Profiles for ATP and Adenosine Release at the Carotid Body in Response to O_2 Concentrations

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1. INTRODUCTION

Excitatory effects on carotid body (CB) chemotransduction have been described for both adenosine and ATP. Adenosine when applied exogenously increases carotid sinus nerve (CSN) discharges in the cat, *in vivo* (McQueen and Ribeiro, 1983) and *in vitro* (Runold et al., 1990). Administration of adenosine and drugs that increase its endogenous levels stimulate ventilation in rats, an effect abolished by the section of CSN and mediated by A_2 receptors (Monteiro and Ribeiro, 1987, 1989; Ribeiro and Monteiro, 1991). In humans, the intravenous infusion of adenosine causes hyperventilation and dyspnoea, an effect attributed to the activation of CB (Watt and Routledge, 1985, Watt et al., 1987; Maxwell et al., 1986; 1987, Uematsu et al., 2000). The excitatory effect of ATP at the CB described by Zhang et al. (2000) in co-cultures of type I cells with petrosal neurons was further supported by the finding that mice deficient in $P2X_2$ showed a markedly attenuated ventilatory response to hypoxia (Rong et al., 2003) and by the detection of hypoxia- evoked ATP release from chemoreceptor cells of the rat carotid body (Buttigieg and Nurse, 2004).

Two metabolic sources of extracellular adenosine, catabolism of ATP by ecto-5'-nucleotidase and adenosine transport by equilibrative nucleoside transporters were demonstrated for the rat CB (Conde and Monteiro, 2004). The hypothesis that both adenosine and ATP contribute to chemosensory activity and that the balance between their extracellular concentrations is dependent on the intensity of the hypoxic stimuli was tested in the present work. We also investigated the role of external calcium mobilization in the release of both mediators at the CB.

2. METHODS

The present work was carried out in *Wistar* rats (250-350g), anaesthetized with sodium pentobarbital (60 mg/kg ip., Sigma). The rats were tracheostomized and breathed spontaneously during surgical procedure. Carotid bodies, superior cervical ganglion (SCG) and common carotid arterial tissue were removed *in*

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situ, under a Nikon SMZ-2B dissection scope and placed in 500 µl of ice-cold 95% O_2+ 5% CO_2 equilibrated medium containing different drugs in accordance with the protocol used. The incubation medium composition was (mM): NaCl 116; $NaHCO_3$ 24; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1.1; HEPES 10; glucose 5.5; pH 7.42. After removal of the tissues, the animals were sacrificed by an intracardiac injection of a lethal dose of pentobarbital in agreement with the directives of the European Union (Portuguese law nº 1005/92 and 1131/97). After 30 min of pre-incubation in hyperoxia (95% O_2 + 5% CO_2) at 37ºC, the CBs, SCG and arterial tissue were incubated during 10 min in normoxia (20% O_2 + 5% CO_2), hypoxia (2%, 5% and 10%O_2 + 5%CO_2) or hyperoxia (95% O_2 + 5% CO_2) in a medium containing erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 2.5 µM, Sigma), inhibitor of adenosine deamination. The effect of extracellular Ca^{2+} on the release of ATP and adenosine from CBs was assessed in normoxia and 10% O_2 in incubation medium with 0 Ca^{2+} and with 10 mM of EDTA. In some experiments the inhibitor of 5'-ectonucleotidase, α,β-methylene-ADP (AOPCP, 100 µM, Sigma) was also added to the incubation medium. After the incubation period, nucleotides were extracted from the incubation medium and aliquots of the neutralized supernatants were kept at –20ºC until subsequent analysis. Adenosine was quantified by reverse-phase HPLC with UV detection at 254nm (Conde and Monteiro, 2004). For ATP quantification 100 µL of the samples were added to 100 µL of luciferine-luciferase (FLE50, Sigma) and to 4 mL of buffer (in mM: HEPES 20; MgCl2 25; Na_2HPO_4 5). The reaction starts when the enzyme is added to the mixture and the samples were analyzed in triplicate, for 1 minute, by bioluminescence using a luminescence counter (Beckham). Data were evaluated using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA) software and were presented as mean ± SEM values. The significance of the differences between the groups' means was calculated by One-Way or Two-Way ANOVA with Dunnett's and Bonferroni multiple comparison post tests, respectively. Values of $P<0.05$ were considered as representing significant differences.

3. RESULTS

Adenosine and ATP extracellular concentrations obtained in basal conditions (20% O_2) were respectively 67.23 ± 5.36 pmol/CB (n=5) and 6.05 ± 0.70 pmol/CB (n=8). The effect of different O_2 concentrations on the release of adenosine and ATP from CBs is shown in Fig. 1. Hyperoxia did not modify the basal release of adenosine and ATP. Both moderate (10% O_2) and intense hypoxia (2% O_2) increased adenosine extracellular concentrations with the maximal effect (60.8 ± 17.7 %) being achieved with moderate hypoxia (Fig. 1). The effect of hypoxia on ATP extracellular concentrations was more pronounced and maximal concentrations of 16.09 ± 2.64 pmol/CB (n=6) were obtained during intense hypoxic conditions.

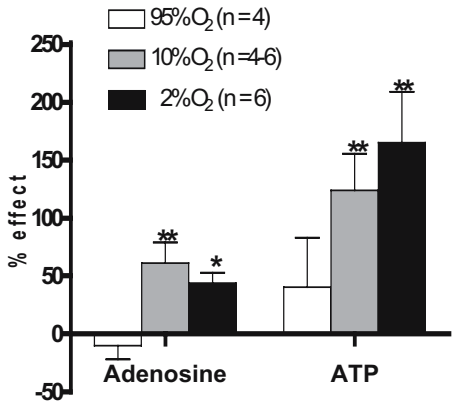

Figure 1. Effect of different oxygen concentrations (2, 10, and 95% O_2) on the release of adenosine and ATP from rat carotid bodies. 0% effect corresponds to concentrations obtained during normoxia (20% O_2). Experiments were performed in the presence of EHNA and 5% CO_2. * P<0.05 and ** P<0.01, One-Way ANOVA with Dunnett's multiple comparison test corresponding to the differences in adenosine or ATP levels between different O_2 concentrations and control conditions (20% O_2).

The effect of different O_2 concentrations (2, 5, 10, 20 and 95%) on the release of ATP from SCG and arterial tissue is shown in Fig. 2. ATP extracellular concentrations obtained during normoxic conditions in SCG and arterial tissue were respectively 16.23 ± 3.61 pmol/mg (n=6) and 24.68 ± 7.42 pmol/mg. No changes in ATP concentrations in response to O_2 were found in arterial tissue (Fig. 2). In contrast, increases in ATP concentrations of about 154% and 298% were observed in SCG during intense (respectively 5 and 2% O_2) hypoxic conditions. Moderate hypoxia did not cause significant increases in ATP concentrations in SCG.

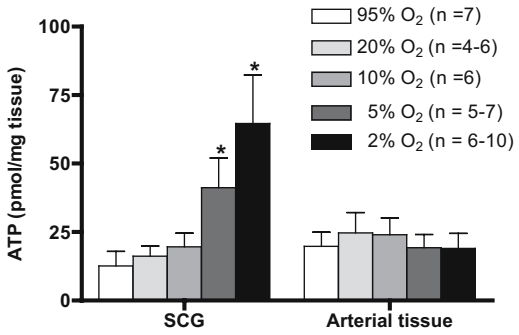

Figure 2. Effect of different oxygen concentrations (2, 5, 10, 20 and 95% O_2) on the release of ATP from superior cervical ganglion (SCG) and carotid arterial tissue Experiments were performed in the presence of EHNA and 5% CO_2. * P<0.05 One-Way ANOVA with Dunnett's multiple comparison test corresponding to the differences in ATP values between different O_2 concentrations and normoxic conditions (20% O_2).

The contribution of extracellular calcium to the mechanisms involved in the release of adenosine and ATP from the CB was assessed in experiments in the presence of EDTA and the absence of Ca^{2+} in the incubation medium. The effects of external Ca^{2+} mobilization in normoxia and moderate hypoxia

(10% O_2) are summarized in Table 1. Removal of extracellular calcium did not modify the basal release of either adenosine or ATP in normoxic conditions but completely abolished the release of those transmitters evoked by moderate hypoxia (Table 1). The reduction in the release of adenosine and ATP in moderate hypoxia caused by the absence of extracellular calcium was of the same magnitude (≈ 50%) for both transmitters.

Table 1. – Influence of external calcium mobilization on adenosine and ATP release from rat carotid body.

	Adenosine (pmol/CB)		**ATP** (pmol/CB)	
	20% O_2	**10% O_2**	**20% O_2**	**10% O_2**
Control	67.56 ± 6.32 (6)	107.1 ± 11.55^{++} (6)	6.05 ± 0.70 (6)	13.21 ± 1.97^{++} (6)
0Ca^{2+} + EDTA	70.73 ±7.30 (4)	53.14 ± 6.43 *** (5)	6.65 ± 1.02 (11)	6.59 ± 0.63*** (13)

Values represent mean ± SEM (n). $^{++}$ P<0.01, compared with 20% O_2 in control conditions; *** P<0.001 compared with 10% O_2 in control conditions (Two-Way ANOVA with Bonferroni multiple comparison test)

In order to investigate whether the amount of adenosine released in hypoxia by the CB through an extracellular Ca^{2+}-dependent mechanism came from primary ATP release and its further catabolism, experiments were performed in the presence of the inhibitor of 5'-ectonucleotidases, AOPCP. In the absence of extracellular calcium, AOPCP reduced adenosine concentrations from 70.73 ± 7.3 pmol/CB to 45.90 ± 5.11 pmol/CB (n=6) in normoxia (*P<0.05). In contrast, no statistically significant differences were found between adenosine concentrations measured in the absence of extracellular calcium in moderate hypoxia, before (53.14 ± 6.43 pmol/CB) and after (42.76 ± 4.37 pmol/CB, n=8) the addition of AOPCP to the medium.

4. DISCUSSION

In response to hypoxia, the CB releases both adenosine and ATP and moderate hypoxia (10% O_2) was a strong enough stimulus to trigger the release of both transmitters increasing the ATP/adenosine ratio with the intensity of the hypoxic conditions.

The magnitude of the effect of moderate hypoxia on adenosine extracellular concentrations at the CB is in agreement with that previously described (Conde and Monteiro, 2004) in the rat but the effect of more intense hypoxic stimulations have never been reported. The release of ATP from the CB was first shown by Buttigieg and Nurse (2004) in the rat *in vitro* in response to intense hypoxic stimulations (15 - 20 mmHg ≈ 2% O_2). In the present work extracellular concentrations of adenosine and ATP were measured in the same CB and in

response to different O_2 concentrations in order to understand the relative contribution of both transmitters to chemosensory activity.

Maximal effect on adenosine extracellular concentrations was achieved with moderate hypoxia suggesting that the contribution of adenosine receptor activation at the CB is probably particularly relevant in these circumstances. Extracellular concentrations of adenosine depend on extracellular catabolism of ATP but are also regulated by bi-directional equilibrative nucleoside transporters. An enhanced uptake activity of these transporters induced by extracellular accumulation of adenosine can explain how adenosine concentrations are higher in moderate hypoxia than in strong acute conditions. In turn, the profile of ATP release showing a direct linear correlation with the intensity of the hypoxic stimulus is in agreement with the CSN discharge pattern in response to oxygen concentrations obtained *in vivo* in mice deficient in $P2X_2/P2X_3$ receptors (Rong et al., 2003). Further comparisons based on the absolute values of adenosine/ATP extracellular concentrations are not possible because all the experiments were performed in the presence of an inhibitor of adenosine deamination. ATP extracellular concentrations quantified in the CB were similar to those found by Cunha et al., (2001) in rat hippocampus slices but are about 10 times lower than those described in CB homogenates in cats (Acker and Starlinger, 1984; Obeso et al., 1986) and in rabbits (Verna et al., 1990).

Since the present experiments were performed in whole CB preparations the cell origin of ATP and adenosine cannot be advanced and the release of ATP from SCG terminals in response to hypoxia can of course contribute to the values quantified in the CB. However, 10% O_2 does not seem to be a strong enough stimulus to induce the release of adenosine (Conde and Monteiro, 2004) and ATP (present) in SCG and arterial tissue.

The results of the present work confirmed that in normoxia, approximately 35% of the adenosine released from the CB comes from ATP extracellular catabolism (Conde and Monteiro, 2004) but also provided evidence that the amount of ATP further catalyzed in adenosine by 5'-ectonucleotidases in normoxia originates from an extracellular Ca^{2+}-independent mechanism.

The increase in the release of both adenosine and ATP triggered by moderate hypoxia was completely prevented by removal of extracellular calcium. From this finding we first advanced with the hypothesis that in moderate hypoxia ATP is released from vesicles and extracellular adenosine comes from its catabolism. Further inhibition of 5'-ectonucleotidases did not reduce adenosine concentrations quantified in the absence of extracellular calcium supporting the hypothesis that actually all adenosine originating in moderate hypoxia from ATP is dependent on vesicular release of the nucleotide.

Independently of the cellular and molecular origin of extracellular adenosine in the CB, the nucleoside accumulation in response to hypoxia supports its excitatory effects on ventilation and together with the activation of ATP-P2X receptors can contribute to CSN responses to hypoxia.

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