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Regulation of Oxidative Stress by Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Mediated by PACAP Receptor

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional peptide that has been shown to be neuroprotective following a diverse range of cell injuries. Although several mechanisms regulating this effect have been reported, no direct evidence has linked PACAP to the regulation of oxidative stress, despite the fact that oxidative stress is a factor in the injury progression that occurs in most models. In the present study, we investigated the plasma oxidative metabolite and anti-oxidation potential levels of PACAP-deficient mice, as well as those of wild-type animals treated with PACAP38. These were assayed by the determination of Reactive Oxidative Metabolites (d-ROMs) and the Biological Anti-oxidant Potential (BAP) using the Free Radical Electron Evaluator system. We also investigated the direct radical scavenging potency of PACAP38 and the functional role of its receptor in the regulation of oxidative stress by PACAP, by using vasoactive intestinal peptide (VIP) and the PACAP receptor antagonist, PACAP6–38. Although younger PACAP null mice displayed no significant effect, greater d-ROMs and lower BAP values were recorded in older animals than in their wild-type littermates. Intravenous injection of PACAP38 in wild-type mice decreased the plasma d-ROMs and BAP values in a dose-dependent manner. These effects were not reproduced using VIP and were abolished by co-treatment with PACAP38 and the PAC1R antagonist PACAP6-38. Taken together, these results suggest that PACAP plays an important role in the physiological regulation of oxidative stress.

Keywords PACAP · PACAP-specific receptor (PAC1R) · Oxidative stress · Aging

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Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamus on the basis of its ability to stimulate adenyl cyclase activity (Miyata et al. 1989). PACAP belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily and exists in two amidated forms, PACAP38 and PACAP27. The physiological effects of PACAP are mediated via three receptor types, a higher affinity PACAP-specific receptor (PAC1R) and two lower affinity VIP/PACAP receptors (VPAC1R and VPAC2R), which display similar affinity for VIP and PACAP (Arimura 1998). The receptors are widely distributed in the body (Vaudry et al. 2000) and play diverse roles not only in the central nervous system but also in other organs and tissues. Numerous in vivo and in vitro studies have suggested that PACAP is involved in the suppression of neural (Uchida et al. 1996; Reglodi et al. 2000; Ohtaki et al. 2006, 2008; Ravni et al. 2006) and other forms of cellular death (Arimura et al. 2005; Gasz et al. 2006; Racz et al. 2007; Mori et al. 2010), the modulation or suppression of immune and inflammatory responses (Delgado and Ganea 2001; Abad et al. 2002; Martinez et al. 2002; Ganea and Delgado 2002), and the dilation of vessels and bronchi (Linden 1999; Groneberg et al. 2006; Ohtaki et al. 2004), as

well as playing a role in psychomotor control (Hashimoto et al. 2001, 2006).

Oxidative stress by reactive oxygen species (ROS) is considered a major mediator of tissue and cell injuries as well as some of the deleterious effects of aging. Excess amounts of the ROS superoxide anion (O_2^-) have been recorded immediately after cerebral ischemia and reperfusion (I/R) prior to a rise in neuronal cell death (Ohtaki et al. 2007). In contrast, mice deficit in the proinflammatory cytokine, IL-1α/β gene, shows less brain infarction than the wild-type counterparts following I/R, and has decreased levels of nitric oxide and 3-nitrotyrosine, a metabolite of peroxynitrite ($ONOO^-$; Mizushima et al. 2002; Ohtaki et al. 2003), whereas transgenic mice in which the manganese superoxide dismutase gene is muted display increased neuronal cell death after I/R (Fujimura et al. 1999; Kawase et al. 1999).

To date, it has been demonstrated that PACAP ameliorates a diverse range of cell injuries, with most in vitro and in vivo injury models involving oxidative stress in the progression of the insult. It has also been reported whether PACAP leads to an increase in the level of the anti-oxidants, peroxiredoxin 2 in cultured cerebellar granule neurons (Botia et al. 2008), and heme oxigenase-1 in isolated guinea pig airways (Kinhult et al. 2001). However, no direct evidence has linked PACAP to the regulation of oxidative stress in vivo. In the present study, we demonstrate that, although young PACAP null mice show no significant differences in oxidative metabolites, aged animals show an increase in the plasma levels of these metabolites. Intravenous injection of PACAP38 in wild-type mice leads to decrease oxidative metabolite levels and an increase in anti-oxidative potential in a dose-dependent manner. However, these effects are not observed following co-treatment with PACAP38 and a PACAP receptor antagonist or following treatment with VIP.

Materials and Methods

Animals and Peptides

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Showa University (#04093, 04096). The PACAP null mouse (C57/B6J strain) has been described previously (Hashimoto et al. 2001), and has been backcrossed for at least ten generations. The study which compared the influence of the PACAP gene on oxidative stress was carried out using littermates ($PACAP^{+/+}$, $^{+/-}$, and $^{-/-}$ mice) from the breeding of $PACAP^{+/-}$ mice. The other animal studies were carried out using C57/BL6 wild-type mice obtained from Charles River Japan (Tokyo, Japan). PACAP38, VIP, and the PACAP receptor antagonist, PACAP6-38, were purchased from the Peptide Institute (Osaka, Japan). The peptides were dissolved at a concentration of 10^{-15}–10^{-6}mol/μL in sterilized saline (in vitro) or in filtered (0.2-μm pore) saline containing 0.1% bovine serum albumin (BSA; in vivo). A solution without peptide was used as a vehicle control.

Determination of Oxidative Stress

Oxidative metabolite levels as a marker for oxidative stress were determined using a commercial kit, involving colorimetric determination of Reactive Oxygen Metabolites (d-ROMs) using Free Radical Electron Evaluator (FREE, Health & Diagnostics, Naples, Italy) following the manufacturer's instructions. Photometric readings were employed to determine the generation of a pink aromatic derivative. Briefly, heparinized plasma samples (20 μL) were dissolved in acetate buffer (pH4.8) with $FeCl_2$ at 37°C. These were then gently mixed, and 20 μL of chromogenic mixture including aromatic alkyl-amine were added. After incubation for 5 min at , the pink aromatic derivative generated was measured at 546 nm. The details of the reaction formula have been described in a previous paper (Ohtaki et al. 2010). The results were expressed as unit. One unit coincided with the oxidative potentials of 0.08 mg H_2O_2/dL.

Assay for Anti-oxidative Potential

Anti-oxidative potential was measured with a commercial kit (Biological Anti-oxidant Potential (BAP) test) using FREE and according to the manual, albeit with minor modification. Briefly, plasma aliquots (10 μL) were mixed with reactive solution and the absorbance determined at 510 nm immediately prior to initiation of the reaction. The mixture was then incubated for 5 min at 37°C, and the post-reaction absorbance of the mixture was measured. Under these conditions, the solution loses color, the intensity of this chromatic change being directly proportional to the ability of the incubated sample to reduce ferric ions to ferrous ions (micromoles per liter). The details of the reactive formula have been described in a previous paper (Ohtaki et al. 2010).

Determination of Oxidative stress in PACAP Null Mice

To compare the effect of PACAP gene deficiency on oxidative stress, $PACAP^{+/+}$, $^{+/-}$, and $^{-/-}$ mice (n=23, 13, and 5, respectively) of various ages (50–208 days old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and heparinized blood samples were carefully taken from the right atrium. These samples were centrifuged at 15,000 rpm for 20 min, after which the plasma supernatant was collected. The plasma samples were then snap frozen in liquid nitrogen and were kept at −80°C until assay.

Determination of Direct Radical Scavenge Potential by PACAP38 (In Vitro)

To determine whether PACAP38 itself has anti-oxidative potential or not, we examined in vitro d-ROMs assay on several concentrations of PACAP38. Normal mouse plasma was obtained from anesthetized male C57/BL6 mice (8–10 weeks old). Several concentrations of PACAP38 (1×10^{-15}, $^{-12}$, $^{-9}$, $^{-6}$, and $^{-4}$mol/L final, n=3–4 for each concentration) were mixed with pre-wormed plasma. Immediately, the plasma containing PACAP was tested with d-ROMs and BAP as described above.

Determination of Indirect Radical Scavenge Potential by PACAP and PACAP6-38 (In Vivo)

Male C57/BL6 mice (86.5±3.3 days old, n=4–6) were transiently anesthetized by inhalation of 3.0% sevoflurane in N_2O/O_2 and gently laid on their back while maintaining rectal temperature at 37–38°C using heat blanket. Initial pre-treatment blood samples (80 μL) containing heparin (5 μL) were carefully collected from the jugular vein (0 h), following which either vehicle (saline containing 0.1% BSA) or PACAP38 solution (5×10^{-11}, $^{-9}$, or $^{-7}$mol/kg of body weight) was injected into the jugular vein (100 μL). Thereafter, the blood samples were collected at 1, 3, 6, 16, and 24 h after PACAP injection. Other animals (n=4 in each group) were injected intravenously with VIP (5×10^{-7}mol/kg), PACAP6–38 (5×10^{-7}mol/kg), or PACAP together with PACAP6–38 (5×10^{-7}mol/kg each). Blood samples were collected pre-treatment (0 h) and at 3 h after the injection. Between sampling points, the animals were recovered from anesthesia and kept freely in a home cage. The heparinized blood samples were centrifuged and the plasma used to carry out d-ROMs and BAP tests.

Statistical Analysis

Data are expressed as mean±SEM. Statistical comparisons were made by one-way ANOVA following Dunnett's post hoc test as compared to the PACAP$^{+/+}$ (wild-type) mice or vehicle-treatment animals. A value of P<0.05 was considered statistically significant.

Results

Effect of Endogenous PACAP and Age on Oxidative Stress

The heparinized plasma obtained from PACAP mutant and wild-type mice (64 to 219 days old) was used to determine oxidative metabolite levels and anti-oxidative potential as a measure of the effect of PACAP on oxidative stress. Figure 1a illustrates the gradual increase in d-ROMs value with age in PACAP $^{+/+}$, $^{+/-}$, and $^{-/-}$ mice (n=23, 13, and 5, respectively), these factors being highly correlated (r^2= 0.741, 0.764, or 0.976, respectively).

The mean d-ROMs value across all ages was greater in the case of the PACAP$^{-/-}$ mice than their wild-type littermates (137.0±10.4 U vs 105.5±3.9 U; mean±SE; p<0.01). The value in the case of younger animals (50–100 days) was similar across genotypes, whereas that of older (150–220 days) PACAP$^{-/-}$ mice was significantly higher than that of their wild-type littermates (146.5±5.4 U vs 123±3.9 U; p<0.01; Fig. 1c).

Next, the correlation between age and plasma BAP values was examined (Fig. 1b). No correlation was found in the case of PACAP$^{+/+}$, $^{+/-}$, or $^{-/-}$ mice, with the correlation coefficient (r^2) in all three cases being less than 0.35. However, when the effects of age were considered (Fig. 1d), the BAP value in older PACAP$^{-/-}$ mice was shown to be significantly lower than that of their age-matched wild-type littermates (3,007±116 μmol/L vs 3286±107 μmol/L; p< 0.01). These results suggest that the absence of PACAP leads to an increase in oxidative stress with aging.

Potential of Direct Radical Scavenging by PACAP38 (In Vitro)

To evaluate the oxidative and anti-oxidative potential of PACAP itself, we examined the effect of several concentrations of PACAP38 in the d-ROMs and BAP tests. As shown in Fig. 2, no significant differences were observed in either assay at any of the concentrations of PACAP38 (1×10^{-15}, $^{-12}$, $^{-9}$, $^{-6}$, and $^{-4}$mol/L). These results suggest that PACAP38 alone has no direct effect but that the increase in d-ROMs value and decrease of BAP value in the plasma of the PACAP$^{-/-}$ mice might mediate an indirect physiological response.

Effect of Exogenous PACAP38 on Oxidative Stress In Vivo

We next determined the effect of exogenous PACAP on oxidative stress in vivo. Vehicle or PACAP38 (5×10^{-11}, $^{-9}$, and $^{-7}$mol/kg) was injected intravenously into the jugular vein of C57/BL6 wild-type mice, and plasma samples were assayed for d-ROMs and BAP in a time-dependent manner (Fig. 3a, b). No significant difference being recorded at 0 h in any of the treated animals. However, after PACAP38 injection, the d-ROMs value began to decrease from 1 h in a dose-dependent manner, with a significant difference being recorded at a concentration of 5×10^{-7}mol/kg 3–6 h later. This was sustained until the 16-h time-point, although the levels did gradually return toward the vehicle-treated values. The d-ROMs value at 3 h was 56.0±11.5 U (62% of 0 h value and p<0.05 vs vehicle).

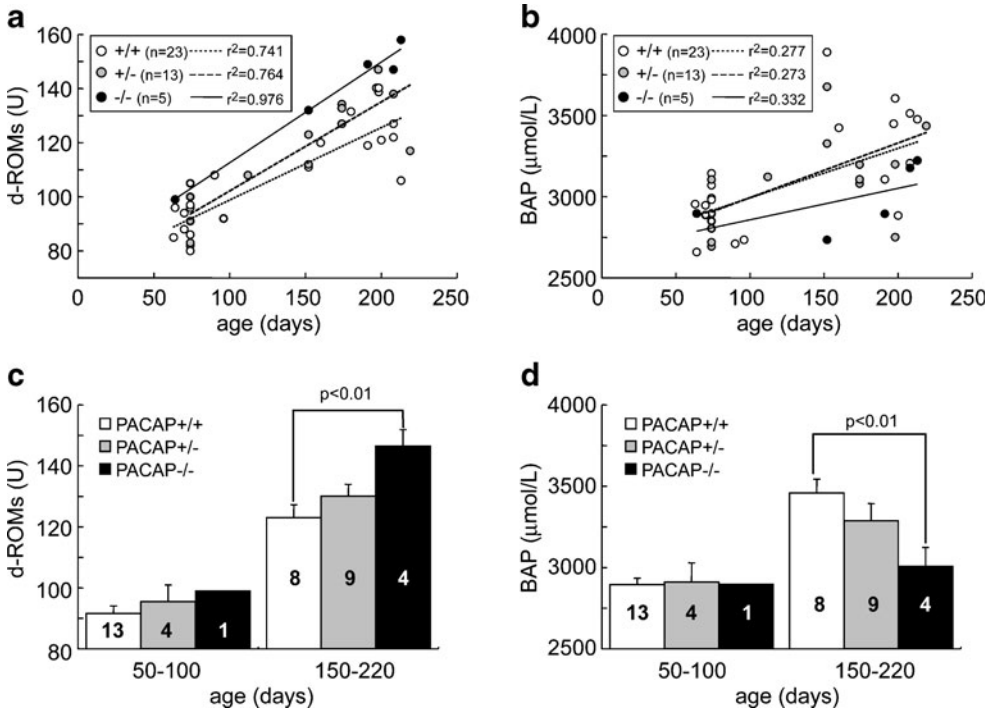

Figure 1 The plasma of aged PACAP null mice displayed increased oxidative metabolites and decreased anti-oxidative potential. Plasma samples of PACAP $^{+/+}$, $^{+/-}$, and $^{-/-}$ mice were analyzed for oxidative metabolites (**a**, **c**) and anti-oxidative potential. Plotting individual d-ROMs (**a**) and BAP (**b**) values revealed that the d-ROMs value was highly correlated with age, whereas the BAP was not. The correlation coefficient (r^2) in the case of the PACAP$^{+/+}$, $^{+/-}$, and $^{-/-}$ mice was 0.741, 0.764, and 0.976, respectively. The r^2 for the BAP value was less than 0.35 in all cases. Mean plasma d-ROMs (**c**) and BAP (**d**) in younger (50–100 days old) or older (150–220 days old) mice revealed significantly greater oxidative stress in the older PACAP$^{-/-}$ animals than in their wild-type (PACAP$^{+/+}$) littermates. Data are expressed as mean±SE. The number of samples is indicated in the columns. $p<0.01$ vs the wild-type (PACAP$^{+/+}$) mice by Dunnett's post hoc test

BAP was also determined in the same plasma samples (Fig. 3b). No significant differences were observed at 0 h with any concentration of PACAP38. However, the plasma BAP following 5×10^{-7}mol/kg PACAP38 treatment was significantly greater than that of vehicle-treated sample at 1–6 h. These results suggest that PACAP decreases oxidative stress.

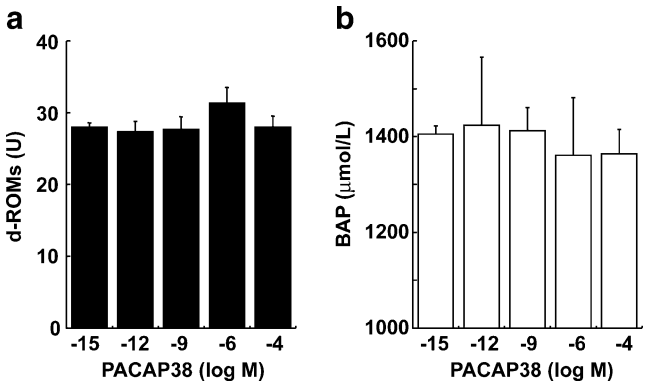

Figure 2 Direct radical modifying effect of PACAP. PACAP38 was prepared at concentrations of 1×10^{-4}–1×10^{-15}mol/L, with these being added in the d-ROMs (**a**) and BAP (**b**) assays to observe direct regulation of oxidative stress. No significant differences were recorded in either assay. Data are expressed as mean±SE (n=3–4 for each concentration)

PACAP Receptors are Involved in the Anti-oxidative Effect on PACAP

Three receptors, PAC1R, VPAC1R, and VPAC2R, have been reported to play a physiological role in terms of PACAP function. PACAP binds to PAC1R with higher affinity than to VIP but binds to VPAC1R and VPAC2R with similar affinity to VIP (Arimura 1998). To determine the functional receptor mediating the anti-oxidative effect of PACAP, we treated mice with VIP, PACAP6-38 (PACAP receptor antagonist), or combination of PACAP38 and PACAP6-38, each at a concentration of 5×10^{-7}mol/kg as part of the PACAP dose response series described above. Plasma samples were then assayed for d-ROMs and BAP (Fig. 3c, d). As shown above, PACAP38 decreased d-ROMs and BAP values 3 h after injection. However, VIP did not influence d-ROMs and BAP values at 3 h. Indeed, the samples in combination of PACAP38 and PACAP6-38 or PACAP6-38 alone also recorded no significant differences

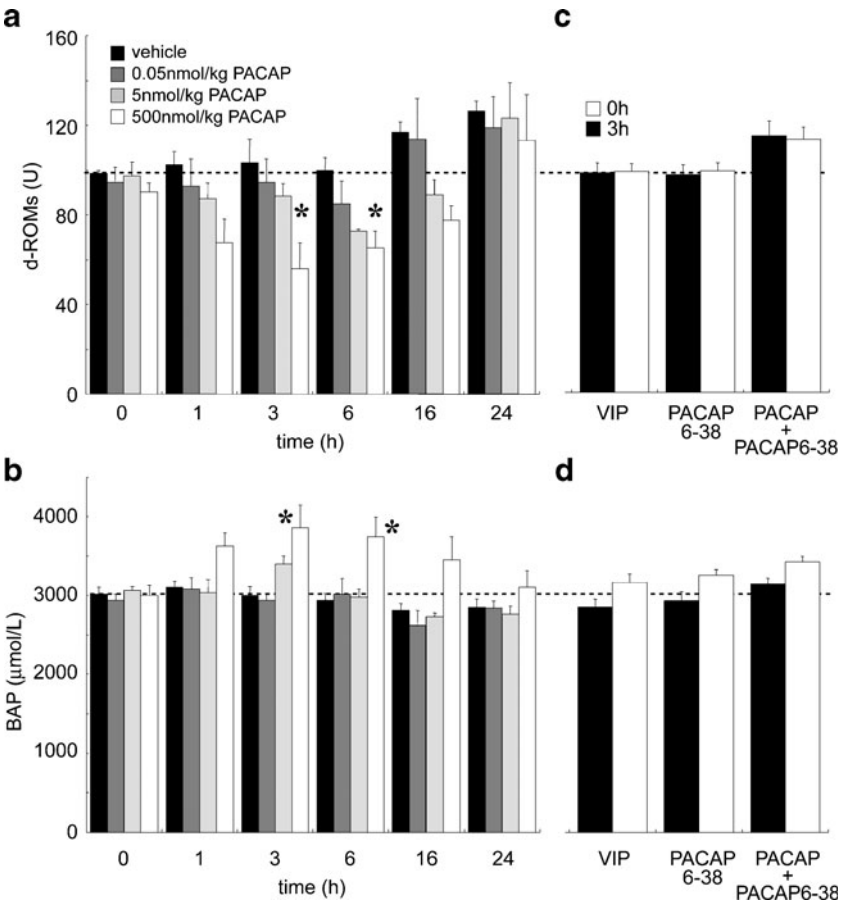

Figure 3 Endogenous PACAP38 regulates the oxidative stress mediated by PAC1R. **a**, **b** PACAP38 at 0.05, 5.0, or 500 nmol/kg (5×10^{-11}, $^{-9}$, or $^{-7}$mol/kg) or vehicle were injected into jugular vein of wild-type mice, and d-ROMs (**a**) and BAP (**b**) were assayed in the plasma for up to 24 h (n=4–6). The plasma of the PACAP-treated mice showed a decreased d-ROMs value and an increased BAP value in a dose-dependent manner. **c**, **d** VIP, PACAP6-38, or PACAP38 together with PACAP6–38 (PACAP + PACAP6–38) were injected into the jugular vein of wild-type mice (n=4 in each group), and d-ROMs (**c**) and BAP (**d**) were assayed. No significant differences were recorded. Data are expressed as mean±SE. *p<0.05 vs vehicle-treated animal by Dunnett's post hoc test

on the d-ROMs and BAP. The results suggested that the effect of PACAP on oxidative stress may be mediated PACAP receptor.

Discussions

It is well known that PACAP protects neuronal cells from diverse insults such as hydroxyl peroxide exposure and ischemia (Vaudry et al. 2002, 2005; Ohtaki et al. 2006, 2008), with recent evidence suggesting that it may also rescue other organs or tissues including kidney (Arimura et al. 2005; Li et al. 2010; Horvath et al. 2010), heart (Gasz et al. 2006; Mori et al. 2010), inner ear (Racz et al. 2010), and endothelial cells (Racz et al. 2007). Most injuries lead to the production of ROS and oxidative stress. However, it has not been determined whether PACAP regulates this oxidative stress, although some of reports have raised the possibility of its involvement in the production of anti-oxidants (Kinhult et al. 2001; Reglodi et al. 2004; Botia et al. 2008). In the present study, we have clearly demonstrated that PACAP produces a physiological response by acting as an anti-oxidant. Moreover, we have also shown that the effect of PACAP is mediated via PACAP receptors and have clarified the contribution of endogenous PACAP with aging. Increases in oxidative stress with aging have previously been established in rodents and humans (Voss and Siems 2006; Rajawat et al. 2009; Chiba et al. 2009). Consisted with these findings, our results revealed a high correlation between increased plasma d-ROMs values and age. When the contribution of the PACAP gene was considered, an increase in d-ROMs value was observed with aging in all cases, although the slope of the correlation curve differed between genotypes. The highest correlation was seen in the case of PACAP$^{-/-}$ mice. Similar plasma d-ROMs values were recorded for younger animals regardless of genetic background, but older PACAP$^{-/-}$ mice had higher plasma levels and lower BAP values than their PACAP$^{+/-}$ mice or wild-type littermates. These results strongly suggest that endogenous PACAP acts as an anti-oxidant in the regulation of oxidative stress. Given that PACAP is known to exist in the circulation, we also examined its direct effect on oxidative stress of PACAP in the circulation (Borzsei et al. 2009) but observed no direct free radical modifying potential of PACAP38. These results, together with the finding that intravenously injected PACAP, decreased the d-ROMs value and increased the BAP value in a dose-dependent fashion, which indicate that the effect of PACAP

on oxidative stress is mediated via physiological responses. We next focused on the functional receptor that mediates the anti-oxidative potential of PACAP. VIP was used as an agonist of VPAC1R and VPAC2R, given that PACAP and VIP share similar affinities for these receptors and PACAP6-38 was used for antagonist of PAC1R and VPAC2R. Injections of VIP or PACAP6-38 alone produced no change in the d-ROMs and BAP values, whereas co-treatment with PACAP38 and PACAP6-38 antagonized the effect of PACAP38. These results indicate that PACAP produces an anti-oxidative response mainly via PAC1R and/or VPAC2R and not via VPAC1R. What still remains unclear is whether the anti-oxidative potential of PACAP mediates protection from cell and tissue damages. An exogenous PACAP38 concentration at 5×10^{-7}mol/kg was required to decrease the d-ROMs value significantly. However, in the rodent, PACAP has been shown to prevent neuronal and renal cell death at 5 nmol/kg (5×10^{-9}mol/kg) or lower (Uchida et al. 1996; Arimura et al. 2005; Ohtaki et al. 2006, 2008). It is therefore possible that the anti-oxidative potential of PACAP that we observed in the present study might not play a critical role in the case of pathological conditions. In contrast, although $PACAP^{-/-}$ mice suffered greater oxidative stress, infusion of PACAP6–38 (5×10^{-7}mol/kg) alone did not influence the d-ROMs and BAP values, suggesting that long-term absence of endogenous might lead to a cumulative oxidative stress. Further studies are required to clarify the anti-oxidative potential of PACAP and its ability to prevent cell death and to demonstrate the specificity and/or diversity of this effect in different tissues.

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