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The effects of recurrent hypoglycaemia and opioid antagonists on the adrenal catecholamine synthetic capacity in a rat model of HAAF

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ABSTRACT

In this study, we investigated the effects of recurrent hypoglycaemia on the adrenal catecholamine synthetic enzymes in a rat model of hypoglycaemia-associated autonomic failure (HAAF). We found that plasma adrenaline was significantly reduced by about 50% in response to recurrent hypoglycaemia versus single hypoglycaemia. However, tyrosine hydroxylase (TH) protein and phosphorylation at Ser31 and Ser40 were increased in HAAF; similarly, aromatic aminoacid decarboxylase protein was also increased indicating a likely increase in catecholamine synthesis in the adrenal gland. Opioid antagonists, naloxone and methylnaltrexone did not restore plasma adrenaline in HAAF; however, naloxone increased TH phosphorylation at Ser31 and Ser40.

1. Introduction

Repeated episodes of hypoglycaemia lead to a condition called hypoglycaemia-associated autonomic failure (HAAF) (Cryer, 2008), characterized by reduced plasma adrenaline response to subsequent hypoglycaemia. Tyrosine hydroxylase (TH) is the rate - limiting enzyme in catecholamine biosynthesis. In the short term, TH is regulated via phosphorylation of three serine (Ser) residues - Ser19, Ser31 and Ser40 and in the long term, via increased protein synthesis (Tekin et al., 2014; Dunkley et al., 2004). When catecholamines are released from the adrenal gland there is a concomitant increase in catecholamine synthesis to replenish the stores via increased TH phosphorylation in the short term and TH protein in the long term. Previously, we found that insulin caused a prominent increase in Ser31TH phosphorylation in the adrenal gland of rats within 60 min of stimulation, in parallel with increased plasma adrenaline levels (Senthilkumaran et al., 2016a). In this study, we hypothesized that catecholamine synthetic capacity of the adrenal gland may be reduced after repeated episodes of hypoglycaemia and this may contribute to the diminished catecholamine release in HAAF. Consequently, our first aim was to determine if the site-specific TH phosphorylation, TH protein and other catecholamine-synthetic enzymes in the adrenal gland are altered in HAAF (Senthilkumaran et al., 2016b).

Recent human studies have demonstrated that administration of the opioid antagonist naloxone during hypoglycaemia has prevented the effect of antecedent hypoglycaemia to diminish adrenaline response to subsequent hypoglycaemia in both non-diabetic individuals (Caprio et al., 1991; Leu et al., 2009) and individuals with type 1 diabetes (Caprio et al., 1991; Vele et al., 2011). The mechanisms of action of naloxone are not known, however, earlier studies demonstrated that opioids can inhibit catecholamine release from the PC12 rat pheochromocytoma cells, bovine adrenal chromaffin cells and rat adrenal glands (Dermitzaki et al., 2001; Jarry et al., 1989; Saiani and Guidotti, 1982; Venihaki et al., 1995) and this inhibition can be reversed by naloxone in some studies (Dermitzaki et al., 2001; Saiani and Guidotti, 1982; Venihaki et al., 1995). Also, opioids (such as beta-endorphins and enkephalin) can be co-released from the chromaffin cells together with catecholamines (Cheng et al., 2001; Livett et al., 1981) and thus, it is possible that the endogenously released opioids may regulate catecholamine secretion from the adrenal gland in an autocrine/paracrine fashion *in vivo* (Jarry et al., 1989). Furthermore, glucose deprivation activates a subset of C1 neurons (Ritter et al., 1998; Verberne and Sartor, 2010) in the rostral ventrolateral medulla which innervate a subset of sympathetic preganglionic neurons (SPN) in the spinal cord (Parker et al., 2013) which in turn innervate adrenaline-secreting chromaffin cells of the adrenal medulla (Morrison and Cao, 2000). Recent studies have shown that a subset of C1 neurons and a subpopulation of SPN, responsible for the release of adrenaline from the adrenal medulla in response to glucoprivation, express the inhibitory opioid enkephalin (Parker et al., 2013; Parker et al., 2015). Hence, it is possible that the opioids may also modulate the adrenaline secretory response to hypoglycaemia at the level of C1 neurons and SPN-adrenal interface. Consequently, our second aim was to investigate if opioid antagonists can alter TH regulation in the adrenal gland and restore the catecholamine secretory response in HAAF in rats. In this study, we used two opioid antagonists, naloxone and methylnaltrexone. Naloxone

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can cross the blood-brain barrier and therefore can act both centrally and at the periphery, while methylnaltrexone does not cross the blood-brain barrier and thus can act only at the periphery. By using these two antagonists we aimed to establish if the effects of opioids in HAAF are mediated centrally or peripherally.

2. Materials and methods

2.1. Animal treatments

All animal experiment protocols were approved by the SA Pathology (Australia) animal ethics committee. Adult male Sprague Dawley rats of 9–12 weeks of age were purchased from Laboratory Animal Services (University of Adelaide, Australia) and maintained in groups of three per cage with access to food and water.

Previously, we tested two HAAF protocols (Senthilkumaran et al., 2016b) involving one or two episodes of antecedent hypoglycaemia per day for 2 days followed by the last episode of hypoglycaemia on day 3. Since we did not observe any difference in plasma adrenaline response between these two protocols (Senthilkumaran et al., 2016b), we adopted the protocol involving one episode of antecedent hypoglycaemic episode per day for the main study (for a review of different protocols see Senthilkumaran et al. (2016b)).

After handling, rats (n = 41) were allocated into either control (Control), Single hypoglycaemia (Single), HAAF, HAAF + methylnaltrexone (HAAF + MNalt) or HAAF + naloxone (HAAF + Nal) groups. We chose to use 10 U/kg insulin (Insulin lispro Humalog, Eli Lilly, Australia) to induce both antecedent and subsequent hypoglycaemic episodes as established previously (Senthilkumaran et al., 2016b).

All drugs were administered by the intraperitoneal route. The control group was injected with saline once a day for 3 consecutive days; the single hypoglycaemia (Single) group was injected with saline once a day on the first two days and with insulin (10 U/kg) on day 3; the HAAF, HAAF + MNalt and HAAF + Nal groups were injected with insulin (10 U/kg) once a day for 3 consecutive days. HAAF + MNalt and HAAF + Nal groups were administered with methylnaltrexone (4 mg/kg; Relistor (Methylnaltrexone bromide), Link Medical Products Pty Ltd., Australia) and naloxone (5 mg/kg; catalogue # 15594, Cayman Chemical Company, USA) respectively 15 min prior to the insulin injection on each day. Tail vein blood glucose was measured at − 5, 30, 60, 90 and 120 min on days 1 and 2 and at − 5, 30 and 60 min on day 3 using a blood glucose meter (Accu-chek Performa, Roche Diagnostics, Germany) as per manufacturer's instructions to ensure that hypoglycaemia was achieved during each hypoglycaemic episode. On days 1 and 2, food was withheld for all rats for 120 min after the saline/insulin injection. In insulin treated rats, blood glucose level stayed between 2 and 3 mmol/L for the duration of 120 min during antecedent hypoglycaemia episodes on day 1 and 2. Rats were overnight fasted prior to day 3. On day 3, rats were euthanized by sodium pentobarbitone injection (1 mL at 325 mg/mL, i.p) at 60 min. One whole adrenal from each rat was rapidly removed and analysed for total TH and phosphorylated TH as described previously (Senthilkumaran et al., 2016a) and below. The second adrenal gland was dissected out, the adrenal cortex was removed using a surgical scalpel and the adrenal medulla was collected and analysed for other catecholamine synthetic enzymes and phosphorylated ERK1/2 as described below. Blood samples were collected through cardiac puncture and plasma adrenaline was measured as previously described using plasma adrenaline ELISA kit (Senthilkumaran et al., 2016a).

2.2. Western blot analysis

Equal amounts of adrenal proteins (after analysis by micro BCA protein assay kit, Thermo Fisher, USA) were separated on SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (Senthilkumaran et al., 2016a). The blots were then incubated with primary total TH (catalogue # T1299, Sigma-Aldrich, USA), pSer40TH and pSer31TH (produced and validated for specificity as described previously (Gordon et al., 2009)), pERK1/2 (catalogue # sc - 16982, Santa Cruz Biotechnology, Inc., USA), total ERK1/2 (catalogue # sc - 93, Santa Cruz Biotechnology, Inc., USA), aromatic amino acid decarboxylase (AADC) (catalogue # sc - 46909, Santa Cruz Biotechnology, Inc., USA), dopamine beta hydroxylase (DBH) (catalogue # sc - 47707, Santa Cruz Biotechnology, Inc., USA) and Phenylethanolamine N- methyltransferase (PNMT) (catalogue # ab69579, Abcam, Australia) overnight at 4 °C followed by 1 h incubation at room temperature with corresponding secondary antibodies; anti-mouse antibody (catalogue # 715-035-150, Jackson ImmunoResearch Laboratories, USA) for total TH, AADC and DBH; anti-sheep antibody (catalogue # 713-035-147, Jackson ImmunoResearch Laboratories, USA) for pSer40TH and total ERK1/2; and anti-rabbit antibody (catalogue # 711-035-152, Jackson ImmunoResearch Laboratories, USA) for pSer31TH and PNMT. The resultant immunoblots were developed using home-made ECL reagents (10 mL of 100 mM Tris HCl (pH 8.5), 22 μL of 90 mM coumaric acid, 50 μL of 250 mM luminol and 3 μL of H_20_2), and the bands were visualised and quantified using ImageQuant LAS 4100 imaging system (GE Healthcare, United Kingdom) as described previously (Senthilkumaran et al., 2016a). The densities of the bands were measured as a fold increase relative to the controls. Site-specific TH phosphorylation for each serine residue was corrected for total TH protein as previously described (Damanhuri et al., 2012; Ong et al., 2011). Phosphorylation for ERK1/2 was corrected for the corresponding total ERK1/2 protein. Total TH, AADC, DBH and PNMT proteins were corrected for β-actin (catalogue # A3584, Sigma-Aldrich, USA) protein to account for variability in protein loading during gel electrophoresis.

2.3. Statistical analysis

Statistical analysis was carried out using PRISM V6.05 (GraphPad Software, Inc., CA, USA). One-way ANOVA with Tukey's post hoc test was used to examine any significant effects of treatment across the groups. All data are presented as a fold increase of the mean ± error bar relative to control group. P value < 0.05 was deemed to be statistically significant.

3. Results

3.1. The effects of repeated insulin injections on blood glucose and plasma adrenaline

Blood glucose level (Fig. 1a) at 60 min was significantly reduced to the same level in both single hypoglycaemia group and HAAF rats (1.7 mmol/L and 1.8 mmol/L respectively; $p < 0.001$). Plasma adrenaline (Fig. 1b) was significantly reduced in HAAF rats (2617 ± 185 pg/mL; $p < 0.05$) compared to single hypoglycaemia rats (4417 ± 594 pg/mL) as measured at 60 min post injection.

3.2. The effects of recurrent insulin induced hypoglycaemia on catecholamine synthetic enzymes in the adrenal gland

In the adrenal gland, total TH (Fig. 1c) and AADC proteins (Fig. 1f) were significantly increased by repeated (HAAF), but not single (Single) hypoglycaemia (1.9 fold; $p < 0.05$ and 2.7 fold; $p < 0.01$ respectively). The phosphorylation of TH at both Ser31TH and Ser40TH was increased to a similar level by single or repeated hypoglycaemia when corrected per total TH protein (Fig. 1d; 3.9 and 3.2 fold; $p < 0.01$ and Fig. 1e; 1.9 fold; $p < 0.05$ and $p < 0.01$ respectively). In contrast, the other catecholamine synthesizing enzymes DBH (Fig. 1g) and PNMT (Fig. 1h) proteins were unaltered by either treatment. Representative blots for AADC, DBH and PNMT are shown in Fig. 1f, g and h respectively.