Intermedin (Adrenomedullin-2): a Potential Protective Role in Human Aortic Endothelial Cells

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Key Words

Intermedin • Adrenomedullin-2 • Adrenomedullin • Human • Endothelial cells • Aortic cells • Apoptosis • Cell viability

Abstract

Background/Aims: Intermedin (IMD) is a novel peptide with significant vasodilator and cardiac protective actions similar to the related peptide adrenomedullin (ADM). Unlike those of ADM the actions and expression of IMD in endothelial cells are poorly characterised. ADM expression can be increased during cardiovascular disease/stress in vitro and in vivo where it may have a role in several cardiovascular protective actions. To characterise IMD mRNA expression cultured human aortic endothelial cells (HAEC) were stressed by removing serum and bicarbonate, and the addition of hydrogen peroxide. The responses were compared to those of ADM mRNA. We also compared the effects of ADM and IMD on caspase activity and cell viability, and investigated if IMD actions could be altered by a CGRP receptor antagonist. **Methods/Results:** Using the cell immunoblot assay, immunoreactive IMD was shown to be secreted by HAEC. IMD mRNA expression

was also detected in HAEC grown in endothelial growth media (but at markedly lower levels than that of ADM). Absence of bicarbonate, a redox-mediated regulator of endothelial response to various stresses, increased IMD mRNA and ADM mRNA expression. However IMD mRNA, but not ADM mRNA, was markedly increased over time in HAEC in conditions of cell stress including incubation with serum-free Dulbecco's modified Eagle's medium (DMEM) and in response to hydrogen peroxide (H₂O₂). These vigorous responses in IMD mRNA expression were further enhanced by incubation in 5% serum in DMEM without bicarbonate, but in a selective manner since ADM expression was suppressed by serum. We also observed that IMD mRNA was markedly increased and ADM mRNA suppressed in HAEC following a period of suspension and replating. Finally, we observed that IMD, like ADM, increased cell viability in HAEC in DMEM without serum but only IMD reduced caspase activity, perhaps via a yet to be defined receptor system. **Conclusion:** HAECs express IMD mRNA and secrete IMD peptide. IMD mRNA expression is markedly dependent on metabolic conditions and is selectively regulated in a contrary fashion to ADM mRNA. IMD mRNA expression in endothelial cells is markedly sensitive to oxidative stress, and IMD peptide itself

has antiapoptotic activity in human endothelial cells. Our data suggest that IMD has a different role to ADM and may perform a protective function in humans.

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Introduction

Gene analysis of mature peptide sequences by two independent research groups identified in mammals a previously unrecognised peptide of the calcitonin/calcitonin gene-related peptide super family, of which adrenomedullin (ADM) is a member. This new peptide was called intermedin (IMD) by one group [1] and adrenomedullin-2 by the other [2]. Like ADM, IMD signals through calcitonin receptor-like receptor/receptor activity modifying protein (CRLR/RAMP) receptor complexes and activates cAMP-dependant pathways.

IMD mRNA is expressed widely in different tissues of the body but more selectively than ADM [3, 4]. IMD, like ADM, has been demonstrated to have actions on the cardiovascular system. IMD mature peptide has vasodilatory and hypotensive actions with potency similar to or greater than ADM [2, 3]. For example, like ADM, IMD decreased blood pressure and increased both heart rate and renal blood flow in conscious rats [5]. In conscious sheep, IMD was found to have similar hemodynamic effects to ADM and likewise to elevate levels of plasma cAMP, renin and natriuretic peptides but to stimulate, rather than suppress, plasma aldosterone levels [6]. ADM and IMD may also have similar pathophysiological roles under some circumstances with both showing protection against myocardial ischemia/reperfusion injury [7] in rat hearts.

In mice IMD is expressed selectively in endothelial cells within some coronary vessels and within endothelial cells of the glomeruli and vasa recta of the kidney [1]. IMD peptide has also been demonstrated in endothelial cells of rat mesenteric arteries [8]. In man immunocytochemical localization of IMD peptide was observed in myocardial and renal tubular cells but not in renal and coronary blood vessels [4]. In a later study using a different antibody, weak immunostaining in human coronary endothelial cells was detected in addition to stronger staining of human vascular endothelial cells of veins in pericardial adipose tissue [8]. In human skin IMD peptide expression was observed in blood vessels but it was predominantly in the smooth muscle layer [9]. These few reports of IMD presence in endothelial cells may indicate selective expression and roles in distinct vascular

beds. Overall, however, published information regarding IMD expression and regulation in human vascular tissues is limited.

ADM has been suggested to be protective against endothelial cell dysfunction, which is considered to be an early event that leads to vascular wall disorders. ADM in endothelial cells is upregulated in various forms of arteriosclerotic disease where it may have a protective role [10]. Although ADM was identified initially as a potent vasodilator peptide it may also regulate cell proliferation [11] and apoptosis of endothelial cells [12]. ADM mRNA is reported to increase in vascular cells from the aorta in response to H₂O₂ [13, 14], increased glucose levels [15] and ATP inhibition [16]. In order to extend these observations, the behaviour of ADM in HAEC under various other forms of cell stress was characterised here including the absence of serum (which causes activation of apoptotic pathways in cells), and bicarbonate removal, which plays a role in controlling intracellular H⁺ [17].

Numerous studies have linked excess reactive oxygen species and resultant oxidative damage with endothelial dysfunction and atherosclerosis in human arteries [18]. Members of the CGRP family may have unique roles in modulating responses to stress by interacting with different affinities to CGRP/RAMP complexes [19]. In cardiomyocytes of adult spontaneously hypertensive rats IMD, (but not ADM) is markedly upregulated [20] as it is in response to oxidative stress [21]. Marked increases of IMD peptide were also observed in the plasma of rats given a nitric oxide inhibitor [21]. IMD may therefore be markedly sensitive to some forms of cardiovascular stress, in contrast to ADM, even though it may be expressed in the cardiovascular system of healthy adults at levels lower than ADM [20]. Characterisation of the responses and expression of IMD in endothelial cells however is lacking.

Although ADM is highly expressed in cultured aortic endothelial cells of many species including man [22], the expression of IMD by human endothelial cells is not well established. We aimed, therefore, first to determine whether IMD mRNA is expressed in HAECs. In addition, we utilised the cell immunoblot assay, which sensitively detects released protein after it is localised to a binding membrane, to determine whether endothelial cells secreted IMD peptide. Further, we also determined whether IMD mRNA expression is altered by various forms of cell stress and compared this to ADM mRNA responses. The conditions included alterations in the duration of incubation, the effects of DMEM with and without added bicarbonate/CO₂, the absence and

presence of serum, addition of H_2O_2 as an oxidative stress and the effects of attaching cells to a culture surface after being in suspension. Finally, using HAECs we compared the effects of IMD and ADM on caspase activity and cell viability, since antiapoptotic activity may be a potential physiological role of these peptides, and investigated if anti-apoptotic actions of IMD could be inhibited by a CGRP receptor antagonist.

Materials and Methods

Cells

Human aortic endothelial cells (HAEC) (Clonetics, CA, USA) were cultured in endothelial cell growth medium (EGM), which consists of endothelial cell basal medium (EBM) supplemented with endothelial growth medium-2 bulletkit reagents (Clonetics) and 2% fetal bovine serum (FBS), on collagen-1 coated plates (Iwaki Glass Works, Chiba, Japan). Large lung cell carcinoma cells, NCI-H460 (American Tissue Culture Collection, Manassas, USA), were grown in RPMI 1640 medium (Gibco BRL, Auckland, New Zealand) with 10% FBS, and human umbilical vein endothelial cells (HUVEC) were grown in EGM.

IMD gene expression was measured in HAEC taken from 2 women and 1 man aged 50-60 years old. Cells at passages 5-9 were used in experiments (in which cells showed good growth rates, the ability to form homogenous cobblestone shaped monolayers and no significant increase in cell size compared to passage 3 cells). HAEC were cultured on 6-well collagen-1 coated plates in EGM with 5% CO_2 . The medium was then changed to Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) containing 0.1% BSA, 4.5 g/L glucose, L-glutamine, sodium pyruvate, 6.0 g/L HEPES without bicarbonate (DMEM-H) in air at 37°C for 1 hour. RNA was extracted from cells growing in EGM at time zero and then at 1, 3, 12, and 24 hours following the addition of fresh DMEM-H. Gene expression studies were also performed in HAEC growing in EBM, and DMEM with HEPES and bicarbonate (DMEM-HB) with 0.1% BSA in 5% CO_2 . The effects of serum were investigated by adding 5% FBS to the respective media. The effects of oxidative stress were studied by adding hydrogen peroxide (H_2O_2) (Sigma-Aldrich) to EBM and EBM with 5% FBS for 12 hours. The effects of suspending HAEC on mRNA expression were investigated by trypsinizing HAEC which had been grown on collagen-1 coated plates, washing and transferring the HAEC to a sterile vial for 1 hour in DMEM-H at 37°C then replating the cells for 3 hours before extracting RNA. Control plates had media changes performed only.

RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Clifton Hill, Vic, Australia) as previously described [23]. Bioinformatically validated primers (Qiagen) including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Qiagen Cat no QT 00079247), ADM (QT00223846) and IMD

(QT00222173) were used for human samples. Rat primers used included GAPDH (QT001996333), vWF (QT00403095), ADM (QT00385637) and IMD (QT 00422954). cDNA from each sample was analysed for expression of the three genes ADM, IMD and GAPDH. ADM and IMD mRNA expression levels were normalised using GAPDH. All PCR reactions were carried out in at least duplicate. Both IMD and ADM had reaction efficiencies >0.9. Controls included a no template control and a negative control.

RNA extraction from rat aorta

Ethical approval was obtained from the animal ethics committee of the Christchurch School of Medicine and Health Sciences. Four adult female Wistar rats were anesthetized with halothane and decapitated. A midline ventral incision was performed and the chest cavity opened. The aorta was dissected and the surrounding connective tissue removed, flushed with physiological PBS and opened longitudinally. It was then snap-frozen on a glass plate on dry ice. The luminal surface and middle layers of the aorta wall were scraped with a scalpel blade. Tissue shavings were placed in RLT lysis buffer and homogenized with a 20-gauge needle. The RLT homogenate was centrifuged and the supernatant removed and added to an equal volume of 70% ethanol. The RNA was then extracted as described above. PCR was performed on a 1:10 dilution of cDNA.

Caspase assay

HAECs were incubated in 24-well collagen-1 coated plates (Iwaki Glass Works, Chiba, Japan). Cells were collected following trypsinization, centrifuged and pellets frozen at -80°C. Immediately before assay the samples were thawed by addition of 100 μ l of buffer (100 mM HEPES with 10% (w/v) sucrose, 1% (w/v) CHAPS, 5 mM dithiothreitol, 10⁻⁴% (v/v) Nonidet P-40 and 50 μ M Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC)). Caspase activity was measured by monitoring the cleavage of DEVD-AMC: the rate of change in fluorescence was monitored at 370 nm (excitation) and 445 nm (emission). The rate of AMC liberation was calculated and results expressed relative to incubations containing untreated HAEC. Caspase activity was determined at 0, 2, 4, 5, 8 and 12 hours incubation in DMEM in preliminary experiments. Five hours incubation duration was selected for the subsequent assay conditions based on the n-shaped time course established in preliminary studies. Caspase activity was determined in HAEC collected before adding DMEM-H and after 5 hours incubation in DMEM-H or in DMEM-H with added ADM (American Peptide Company Inc, Sunnyvale, CA, USA) (0.01, 0.1, or 1 μ M), IMD (1-53) (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) (0.01, 0.1, or 1 μ M), CGRP (8-37) (AnaSpec Inc, San Jose, Inc) (1 μ M), CGRP (8-37) (1 μ M) with IMD (0.1 and 1 μ M), and 5% FBS. Caspase activity was also determined after 5 hours in HAEC incubated in DMEM-HB, EBM, EBM/5% FBS and EBM/5% FBS with added H_2O_2 (0.025, 0.25, 0.75 mM)

Cell viability assay

HAEC were grown in 24 well plates in EGM. After reaching 90% confluence the cells were washed in fresh media that were

Fig. 1. Levels of ADM mRNA (cross hatched bars) and IMD mRNA (solid bars) after incubation of (A) human aortic endothelial cells (HAEC) in EGM (n=4), (B) human umbilical vein endothelial cells (HUVEC) in EGM (n=3) or (C) large lung cell carcinoma NCIH460 cells in RPMI plus 10% serum (n=2). The levels are expressed relative to the level of ADM mRNA in HAEC incubated in EGM. Data are depicted as mean $\pm$ SEM. *** $p < 0.001$.

to be investigated or in fresh EGM in control wells. After 12 hours, the media were replaced with fresh DMEM-H containing 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) and incubated at 37°C. After a further three hours the media were removed and the crystals that had formed in cells were dissolved in 250 μ l of isopropanol containing 10% dimethyl monosulphoxide. Cell debris was removed from the lysate by centrifugation and absorbance read at 570 nm.

Cell Proliferation Assay

A BrdU proliferation ELISA assay (Roche, Penzberg, Germany) was performed according to the manufacturers instructions. HAEC were grown in 96 well collagen-1 coated plates in the presence of EGM until they reached confluence. The HAEC were washed in DMEM-H then media was replaced with fresh DMEM-H only or DMEM-H containing ADM (1 μ M), IMD (1 μ M) or 5% FBS. After 2 hours incubation BrdU was added to each well to give a final concentration of 10 μ M and cells incubated for an additional 10 hours. Media was removed from cells which were subsequently fixed and incubated for 90 minutes with peroxidase labelled Anti-BrdU. After washing, substrate was added for 20 minutes and absorbance read at 370 nm (ref wavelength 492 nm). Control wells included no cells and no added BrdU.

Determination of cells secreting IMD peptide

The method was based on that previously described [24]. Briefly, HAEC were transferred to glass cloning rings sealed to an Immobilon-P protein binding membrane and incubated in DMEM-H, EBM 0.1% BSA only or EBM with either 0.025, 0.25, or 0.75 mM H_2O_2 for 12 hours. Cells were then fixed with 2.5% glutaraldehyde. The membrane was blocked with 3% BSA for 1 hour. Rabbit anti-IMD antiserum at 1/2000 was incubated with the cells overnight at 4°C and then washed 3x in phosphate buffer (pH 7.4). Rabbit anti-IMD antiserum at 1/2000 was demonstrated to have high affinity for IMD 1-47 and IMD 1-53, but did not cross react with rat ADM, human ADM, calcitonin group related peptide (CGRP), atrial natriuretic peptide (ANP), or brain natriuretic peptide (BNP). Secondary antibody coupled to biotin (anti-rabbit-IgG-biotin, Sigma-Aldrich) in PBS containing 0.3M NaCl was added at 1/1000 dilution to the cells for 1h and then washed 3x in PBS. IMD was visualized with Vectorstain kits (Vector Laboratories) using alkaline phosphatase. Staining localized on the membrane around a cell indicated secretion of IMD by that cell. Numbers of HAEC secreting immunoreactive IMD were counted in 10 rings over 2 separate experiments after both 1 and 12 hour incubations in

DMEM-H. Numbers of HAEC secreting IMD were further studied by incubation with H_2O_2 for 12 hours. 9 rings were counted 3 separate times. Over 150 cells were examined for secretory activity per ring. Control incubations using non-immune rabbit serum (Sigma), only secondary antibody, or anti-IMD serum preabsorbed with IMD peptide (1-53) were examined to account for non specific binding.

Statistics

Statistical analysis was performed using ANOVA or the paired t-test as appropriate. Results are reported as mean $\pm$ SEM.

Results

IMD mRNA in HAECs

We first investigated expression of IMD mRNA relative to ADM mRNA in HAECs cultured in EGM. For comparison we measured the levels of expression in two other human cell types, namely large lung cell carcinoma NCI H460 cells and HUVEC. We used the level of ADM mRNA in NCI H460 cells as a low level reference because expression in those cells is undetectable by northern blotting [25]. IMD mRNA was detectable in HAECs, although the levels were low and ADM mRNA was greater than 30 fold higher ($p < 0.001$, Figure 1). In HUVEC, as in HAECs, lower levels of expression of IMD mRNA, compared to ADM mRNA, were observed ($p < 0.001$, Figure 1). The levels of IMD mRNA in HAEC were low but similar to those of ADM mRNA in NCI H460 cells (Figure 1).

Effect of duration of incubation in DMEM-H on IMD mRNA expression

We next investigated the effects on IMD and ADM mRNA of selected incubation periods in DMEM-H. ADM mRNA levels rose 3.5 ± 0.5 fold ($p < 0.001$) over 12-24 hours incubation compared to levels at time 0 (Figure 2).

Fig. 2. The effect of incubation time in serum-free DMEM-H on mRNA levels of adrenomedullin (ADM) (filled circles) and intermedin (IMD) (filled squares). HAECs were washed and pre-incubated in DMEM for 1 hour. The media were then replaced with fresh DMEM-H, this point being called time 0 hours. Incubation for 12-24 hours in the medium increased ADM mRNA ($p<0.001$) and IMD mRNA ($p<0.01$). The rise in IMD mRNA was significantly greater than that of ADM mRNA ($p<0.01$). Each time point represents at least three separate experiments. mRNA levels are expressed relative to levels at time 0 hours. Data are depicted as mean $\pm$ SEM.

By contrast IMD mRNA levels rose 22.4 ± 5.8 fold ($p<0.01$) over the same period. This rise in IMD mRNA was significantly greater than that of ADM mRNA ($p<0.01$) (Figure 2).

The effect of different media on gene expression

We compared IMD and ADM mRNA expression in HAEC (following a change of medium from EGM) after incubation for 12 hours in EBM, in DMEM-H, or in DMEM-HB. Neither IMD nor ADM mRNA showed a significant increase 12 hours after the medium was changed from EGM to EBM (fold increase relative to EGM 1.2 ± 0.6 and 1.2 ± 0.3 respectively) (Figure 3). Both IMD and ADM increased significantly when the medium was changed to DMEM-H (fold change 20 ± 5.1 $p<0.01$, $n=4$ and 2.8 ± 0.4 $p<0.02$, $n=4$ respectively) (Figure 3). When the medium was changed to DMEM-HB again IMD was relatively more responsive than ADM and IMD mRNA, but not ADM mRNA, was significantly increased (fold change 11.8 ± 3.3 $P<0.05$ and 1.24 ± 0.14 ns respectively) (Figure 3).

The effect of serum on gene expression

HAEC were incubated in DMEM-H with or without 5% FBS for 3 and 12 hours to investigate whether the

Fig. 3. The effects of different cell media without serum on the expression of ADM and IMD mRNA in HAEC at 12 hours. Levels are expressed relative to those observed in EGM at 12 hours. ADM mRNA was not significantly altered in EBM ($n=3$) or DMEM-HB but was raised in DMEM-H ($p<0.05$ $n=6$). IMD mRNA was not altered in EBM but was increased in DMEM-HB ($p<0.01$, $n=4$) and DMEM-H ($p<0.05$, $n=6$). * $p<0.05$; *** $p<0.01$.

Fig. 4. The effect of serum (FBS) and incubation duration on mRNA levels of adrenomedullin (ADM) and intermedin (IMD). Levels of ADM mRNA (cross hatched bars) and IMD mRNA (solid bars) were determined after 3 hours and 12 hours in DMEM-H, and 3 hours and 12 hours in DMEM-H plus 5% FBS. mRNA levels are expressed relative to level of ADM mRNA in DMEM-H at 3 hours. ($n=4$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data are depicted as mean $\pm$ SEM.

levels of ADM and IMD mRNA were modified by serum factors. Data were expressed relative to ADM mRNA levels in HAEC. In the absence of FBS, IMD mRNA levels were lower than those of ADM at both 3 and 12

Fig. 5. Actions of hydrogen peroxide on ADM and IMD mRNA expression in HAEC. IMD mRNA (solid bars) but not ADM mRNA (cross hatched bars) expression was increased significantly in HAEC exposed to H_2O_2 . HAEC were incubated in EBM (a) and EBM with 5% FBS (b) and H_2O_2 (0.025, 0.25 and 0.75 mM) was added to the media for 12 hours. Control incubations were HAEC in which the culture media was changed to EBM or EBM with 5% FBS but without added hydrogen peroxide. ADM mRNA expression in HAEC was not altered significantly by adding H_2O_2 to either EBM or EBM with 5% FBS. IMD mRNA expression was not altered significantly by 0.025 mM H_2O_2 but was increased by 0.25 mM H_2O_2 3.3±0.5 ($p<0.01$) fold and 4.7±1.2 ($p<0.05$) fold in EBM and EBM with 5% FBS respectively. A greater increase in IMD mRNA was seen in HAEC incubated with 0.75 mM H_2O_2 20.7±3.6 ($p<0.01$) fold and 27.1±8.2 ($p<0.05$) fold in EBM and EBM with 5% FBS respectively. The data (mean±SEM) were obtained from 4 separate experiments. * $p<0.05$, ** $p<0.01$.

hours ($p<0.001$ and $p<0.05$ respectively, Figure 4). In the presence of FBS, IMD mRNA levels were similar to those of ADM at 3 hours (Figure 4) but were much higher at 12 hours (9.9±3.0 fold, $p<0.01$). When 5% FBS was added to EBM, a relatively small (2.6±0.3 fold) increased expression of IMD mRNA ($p<0.02$) compared to that in EGM was seen while ADM was decreased in HAEC (0.60±0.001, $p<0.001$) when incubated in EBM with 5% FBS compared to EGM. These data indicate differential regulation of ADM and IMD by factors in serum.

Oxidative stress

We investigated the effect of an oxidative stress using 0.025, 0.25 and 0.75 mM H_2O_2 , on IMD mRNA, and ADM mRNA expression. IMD but not ADM mRNA was dose dependently increased in HAEC by H_2O_2 added to EBM plus 5% FBS and to EBM only (Figure 5). In HAEC incubated in EBM only, IMD mRNA expression was not increased by 0.025 mM H_2O_2 but was increased by 0.25 mM H_2O_2 (3.3±0.5 fold, $p<0.01$) and 0.75 mM H_2O_2 (20.7±3.6 fold, $p<0.01$). Similarly in EBM/5% FBS, IMD mRNA expression was not increased by 0.025 mM H_2O_2 but was increased by 0.25 mM H_2O_2 (4.7±0.1.2 fold, $p<0.05$) and 0.75 mM H_2O_2 (27.1±8.2 fold, $p<0.05$) (Figure 5). Addition of H_2O_2 did not induce a significant change in ADM mRNA expression under any of these conditions (Figure 5).

ADM and IMD mRNA in endothelial cells replated after suspension

We investigated whether changes in HAEC cell contacts and surface adhesion altered ADM and IMD

Fig. 6. Effects of removing cell contacts and adhesion on ADM and IMD mRNA expression. HAEC were removed from collagen coated plates (with trypsin + EDTA) and left in suspension in DMEM-H for 1 hour at 37°C. After this time cells were replated at low density and left to attach for 3 hours in DMEM-H before the extraction of RNA. ADM mRNA levels decreased significantly by 0.4±0.2 ($p<0.02$, $n=4$) fold whereas IMD mRNA levels increased by 7.4±3.1 fold ($p<0.05$, $n=4$). * $p<0.05$; ** $p<0.02$. Data are depicted as mean ± SEM.

mRNA expression during cell dispersion and suspension. It was found that HAEC exhibited reduced ADM mRNA expression (0.4 fold±0.2, $p<0.02$) when cells in DMEM-H were removed from the culture plate and left in suspension for 1 hour then replated for 3 hours at a seeding density (1.2×10^5 cells /35 mm well) in which HAEC were dispersed singly. By contrast, under these conditions IMD mRNA expression increased 7.4 fold ± 3.1 ($p<0.05$) (Figure 6).

Fig. 7. Caspase activity in HAEC was expressed relative to that in HAEC incubated in DMEM-H for 5 hours. Adding bicarbonate to DMEM-H and incubating in 5% CO₂ did not alter caspase activity. However 5% FBS reduced caspase activity $31 \pm 5.8\%$ ($p < 0.001$, $n=7$). Caspase activity was significantly lower with EBM than DMEM-H by $57 \pm 4.6\%$ ($p < 0.001$, $n=4$). Caspase activity was higher with EBM than EGM and EBM with 5% FBS ($p < 0.05$). H₂O₂ 0.75 mM increased caspase activity relative to control ($p < 0.001$, $n=3$). * $p < 0.05$, *** $p < 0.001$. Data are shown as mean $\pm$ SEM.

Caspase activity in HAEC exposed to hydrogen peroxide and serum free conditions

We measured caspase activity in HAEC under various conditions to see if trends of caspase activity reflected changes in IMD mRNA expression. Previously we had observed (Figures 3 and 4) that HAECs incubated in DMEM-H and DMEM-HB had increased IMD mRNA expression compared to cells in EBM. Caspase activities were found to also be higher under these conditions ($p < 0.001$) (Figure 7). HAEC caspase levels were also higher after incubation in DMEM-H with 5% FBS compared to EBM ($p < 0.05$). However 5% FBS reduced caspase activity when added to DMEM-H ($p < 0.001$) or EBM ($p < 0.05$). HAEC caspase levels were similar after incubation in EGM and EBM with 5% FBS. HAEC caspase levels were increased when 0.75 mM H₂O₂ but not 0.025 or 0.25 mM H₂O₂ was added to EBM with 5% FBS (Figure 7).

Cell survival and antiapoptotic activities of IMD and ADM

It has been proposed that ADM may have cell survival activity. Additionally, our results (above) suggest that IMD may be upregulated under conditions of metabolic stress, perhaps (teleologically) with a view to

Fig. 8. Effects of ADM and IMD on caspase activity of HAEC incubated in DMEM-H for 5 hours. ADM had no effect on caspase activity in HAEC but IMD decreased caspase activity at 0.01-1 μ M. CGRP 8-37 did not alter IMD (0.1, 1.0 μ M) actions on caspase activity. The significance symbols indicate probability calculations relative to "Basal" conditions i.e. HAEC incubated in DMEM-H for 5 hours. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are depicted as mean $\pm$ SEM.

enhancing endothelial cell survival. We therefore next investigated the effects of IMD and ADM on cell survival, first by adding IMD, ADM and CGRP 8-37, a CRLR/RAMP-1 and CRLR/RAMP2 receptor antagonist, to HAECs and determining caspase-3 expression and cell viability. CGRP 8-37 was also added to selected incubations with IMD. Second, the effects of IMD and ADM peptides were studied on the proliferation and viability of HAECs, using BrdU incorporation and using the MTT assay respectively.

With regard to the antiapoptotic potential of the two peptides, HAECs were incubated for 5 hours in DMEM-H alone or with added IMD or ADM. IMD (0.01-1 μ M) dose dependently suppressed caspase activity ($p < 0.001$), whereas ADM had no significant effect (Figure 8). CGRP 8-37 (1 μ M) did not inhibit the antiapoptotic actions of IMD (0.1, 1 μ M).

Further, we investigated the effects of IMD and ADM on cell proliferation and cell viability. HAECs were incubated in DMEM-H for 12 hours alone or with added IMD or ADM. Neither ADM nor IMD (1 μ M) altered HAEC proliferation in DMEM-H (Figure 9A). Both peptides significantly increased cell viability, by $11.0 \pm 2.8\%$ ($p < 0.001$) and $6.1 \pm 1.6\%$ ($p < 0.01$) respectively (Figure 9B).

Fig. 9. BrdU incorporation, as a marker of cell proliferation in HAECs incubated in DMEM-H with added IMD (1 μ M) (solid bars) or ADM (1 μ M) (hatched bars) for 12 hours, was determined and expressed relative to BrdU incorporation in HAEC in DMEM-H alone. There was no significant change in cell BrdU incorporation with the addition of either ADM or IMD. Data are depicted as mean $\pm$ SEM. Cell viability of HAECs incubated for 12 hours with 1 μ M ADM and 1 μ M IMD was determined by MTT assay and expressed relative to viability in HAEC in DMEM without added peptide. Both ADM and IMD increased cell viability at 12 hours, by 6.1 ± 1.6 % ($p < 0.01$) and 11.0 ± 2.8 % ($p < 0.001$) respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with basal at 12 hours without added ADM or IMD. Data are depicted as mean $\pm$ SEM.

HAECs secrete IMD

Using the cell immunoblot assay which detects secretion of peptide from individual cells, we observed that, although there was no detectable secretion of IMD at 1 hour, immunoreactive (ir) IMD peptide was released by 12 ± 2 % of HAEC after overnight incubation in DMEM-H relative to control serum (cells in 10 separate rings were counted for secretion over 2 separate experiments, >150 cells were counted per ring).

Fig. 10. This photograph was taken at 100x magnification. The membrane with HAECs was incubated with antiIMD serum (1/2000) and immunohistochemically stained. Arrows show cells surrounded by halos of released irIMD peptide. Photograph taken at 100x magnification. The membrane with HAECs was incubated with antiIMD serum (1/2000) which was preincubated with IMD peptide (absorption control). Arrows show cells in which no peptide release can be seen. This figure shows the percentage of HAEC secreting irIMD with different concentrations of H₂O₂. >150 cells were counted in each of 9 separate rings over three separate experiments. *** $p < 0.001$ compared to control (no H₂O₂). Data are shown as mean $\pm$ SEM.

Next we counted the percentage of HAEC secreting irIMD after 12 hours exposure to 0, 0.025, 0.25, and 0.75 mM H₂O₂ in EBM (0.1% BSA). There was 10.1 ± 2.3 , 14.8 ± 2.2 , 20.1 ± 3.8 , and 52.7 ± 6.6 % of HAEC secreting irIMD respectively at these times. The increased number of HAEC secreting irIMD with 0.75 mM H₂O₂ was significant relative to basal 12 hours and pre-adsorbed control ($p < 0.001$) (Figure 10).

Expression in whole tissue

IDM and ADM mRNA expression were measured in rat aorta to determine whether gene expression occurs in whole tissue from healthy rats. Cells from the deeper layer had 0.18 ± 0.03 times the levels of von Willebrand Factor mRNA found in the luminal layer indicating that most endothelial cells were present in the shallow shavings. Aortic tissue from the luminal surface of the aorta of rats had detectable levels of ADM mRNA but not IDM mRNA. Cells from deeper within the aortic wall also exhibited measurable ADM mRNA but again, not IDM mRNA.

Discussion

IMD is a recently discovered peptide that has been detected in blood vessels of both rodents and humans [8] using immunohistochemical approaches. We report here for the first time that IDM mRNA is expressed in cultured human aortic endothelial cells and compare regulation of IMD message with that of the related peptide, ADM. We found that IDM mRNA was expressed in both HAECs and HUVECs during culture in EGM, a medium that is favourable for endothelial cell growth and viability. The level of expression, however, was markedly lower than that for ADM mRNA. IDM mRNA, in contrast to ADM mRNA, was not detected in the endothelial cell fraction and smooth muscle cell layer of the rat aorta. These data from rat aorta are, in concert with our observation of low IMD expression in HAEC cultured in EGM and with reports that IMD plasma levels are lower than ADM in rats [21].

Although IDM mRNA expression was very low in HAEC cultured in EGM it showed a marked increase when HAEC were transferred to DMEM-H. Of note, the increase in IMD message was significantly more pronounced than that of ADM mRNA. This observation may suggest an enhanced response of IMD compared to ADM to stress i.e. in a less favourable culture medium or environment.

Recent studies showed that that IDM exhibits a marked response in animal models of physiologic stress *in vivo* [20, 21]. In this study we undertook an *in vitro* investigation using human cells in defined environments of metabolic stress and observed differential regulation of IDM and ADM. We first investigated whether the absence of bicarbonate/CO₂ from the culture environment could partly explain, or contribute to, the vigorous increase in IDM mRNA expression seen during incubation of cells

in DMEM-H. Both IDM and ADM mRNA were reduced by adding bicarbonate to DMEM-H (DMEM-HB) and incubating in 5% CO₂. Increased gene expression with bicarbonate removal may represent sensitivity to a particular pathway of cell damage and/or redox state because bicarbonate removal increases the mitochondrial dependent pathway of apoptosis during ischemia-induced apoptosis [17] and is required for intracellular pH homeostasis in endothelial cells under oxidative stress [26]. Nevertheless, IDM mRNA was still more abundantly expressed in cells in DMEM with and without bicarbonate relative to EBM. Second, we observed that IDM mRNA expression in HAEC in DMEM-H was enhanced further by adding serum, whereas in contrast ADM mRNA expression was halved. In this regard, concentration-dependent suppression by FBS of ADM protein release from rat aortic endothelial cells has been previously reported [27]. Although the nature of the serum factors that induced these contrasting effects remains to be determined, serum factor(s) appear to further enhance pathways leading to upregulation of IDM mRNA under stressful conditions only, while ADM expression was suppressed by serum under all conditions. Third, we also report that HAEC, placed in suspension for an hour before replating in serum-free DMEM-H, differentially modulated expression of ADM and IDM mRNA. ADM mRNA levels were significantly reduced, but in contrast IDM mRNA levels showed a substantial increase. Our results suggest that cell detachment and cell-cell communication may contribute to expression of IDM and ADM mRNA but with contrasting effects on the two peptides. IDM mRNA was also more rapidly increased in HAEC exposed to reduced cell attachment in DMEM-H than confluent adherent cells. In this regard, endothelial cells in suspension have rapid increases in oxidative damage [28].

The association that was found between high caspase levels and marked increases in IDM mRNA expression may suggest that caspase-related transcription factors can control IDM mRNA expression in HAEC. With this in mind, analyses (Match™ [29]) of the ADM and IDM promoter regions were performed to look for evidence of stress related transcription factors which may account for differential expression of IDM and ADM. Approximately 75% of putative transcription binding sites of the IDM promoter region were also present in the ADM promoter region. A notable exception to this is a possible c-Myc/Max binding site (aaaCACATgctc) in the IDM promoter region. c-Myc mediated apoptosis is reported to involve c-Myc/Max interactions [30] and be

mediated by cytochrome c release from the mitochondria [31]. Our study revealed that the addition of serum altered the relationship between IMD gene expression and caspase activity levels within cells by further increasing IMD expression without increasing caspase levels. c - Myc is upregulated by serum [32] and accelerates degradation of ADM mRNA [33]. Further ADM mediated anti-apoptotic action in rat aortic endothelial cells occurs by upregulating Max when c-Myc expression is low due to 24 hours serum deprivation [32]. The possibility that binding of the c-Myc/Max heterodimers to the IMD promoter region accounts for at least part of the differential expression of these related peptides awaits confirmation.

Our early studies suggested that IMD would be highly sensitive to an oxidative stress. The subsequent experiments revealed a marked concentration-dependent response of IMD mRNA to H₂O₂-induced oxidative stress in human endothelial cells, whereas, by contrast, ADM mRNA was unaltered. Levels of IMD, but not ADM, have also been observed to markedly increase in both plasma and cardiomyocytes of rats following oxidative stress [21]. Although previous studies found that bovine aortic cells cultured in DMEM had increased expression of ADM when exposed to H₂O₂, possible differences between species, methodology (single northern blots) and the modest increase of ADM (less than 50% over 24 hours) makes interpretation difficult. Our study indicates that IMD responses occurred at concentrations of H₂O₂ (>200 µM) which induce activation of apoptotic pathways and pathways which reduce cell junctions in aortic endothelial cells [34].

In the light of these alterations in expression that were consistent with stress-induced responses (i.e. following extended duration of incubation, incubation in the absence of bicarbonate/CO₂, absence of serum, or addition of H₂O₂ and with cells recently attached to a culture surface after being in suspension) we evaluated, in two separate protocols, the possibility that IMD may play a functional role in processes related to cell survival, including apoptosis. The fact that IMD mRNA and ADM mRNA were both upregulated in DMEM-H in this initial study may reflect a previously-reported cell survival function for the respective peptides to oxidative stress and in serum free media respectively [35, 36]. Further, we demonstrated, for the first time, that IMD (1-53) reduced caspase activation in HAEC, revealing a pathway by which IMD may exhibit antiapoptotic activity. These observations justify further study of the expression of IMD in association with other cell survival processes.

If indeed IMD is an inducible vascular antiapoptotic factor it may play a role in limiting or ameliorating endothelial dysfunction, which is believed to be an early event leading to subsequent vascular disease [37]. In this regard, IMD has been shown to protect both endothelial cells against oxidative damage in vitro [36] and the aorta in vivo in hypertensive rats [38]. We found ADM, in contrast to IMD, did not reduce caspase activation in DMEM-H. Therefore enhanced cell viability of HAECs induced by IMD and ADM peptides may occur through distinct, separate mechanisms. Although ADM has been reported to stimulate endothelial cell proliferation [39] we did not see significant increased proliferation induced by either peptide here in bicarbonate-free conditions. The increased viability of HAEC exposed to ADM, which was less than IMD, may be due to relatively slow effects on viability, or could be due to caspase-independent events. ADM has greater agonistic potency via CRLR/RAMP2 and CRLR/RAMP3 than IMD but possibly not CRLR/RAMP1 [2]. However in this study the presence of the receptor antagonist CGRP (8-37), which strongly inhibits CRLR/RAMP1 [40], did not alter IMD caspase-lowering activity. IMD has been reported to have a distinct receptor in rat spinal cord [41] and pituitary cells [42]. Likewise, in our study, IMD may act to induce anti-apoptotic activity through a receptor system different to that of other members of the CGRP family. Nevertheless the involvement of yet to be delineated interactions with receptors [43] that are activated by members of the CGRP family has not been excluded. Thus IMD may be a factor that is triggered in a pro-apoptotic environment where the peptide modifies the cellular response in order to increase endothelial cell survival by pathways that enable a distinct response to be produced.

Using the cell immunoblot assay that is a sensitive and specific method to detect peptide secretion from individual cells [23, 24], we observed, for the first time, time and dose dependent IMD peptide release from human endothelial cells. As for IMD mRNA, 0.75M H₂O₂ elicited significant IMD peptide responses in HAEC. Although the peptide was not isolated, the specific detection of immunoreactive IMD provides good evidence for the presence of IMD peptide in cultured cells from the human aorta.

Little is known about the regulation of IMD and its possible pathophysiological roles in humans. We conclude from the present studies that IMD mRNA and IMD peptide are expressed in and produced by cultured human endothelial cells. We show that expression of IMD mRNA is increased during incubation in DMEM-H. Gene

expression levels of the two peptides can, however, be differentially modulated as demonstrated by their contrary responses during incubation of HAEC in cell culture media following the addition of FBS, bicarbonate or H₂O₂ and by cells following suspension. IMD, but not ADM mRNA expression, was markedly sensitive to an oxidative stress in endothelial cells. IMD peptide, but not ADM, added to HAECs, reduced caspase activation, implying antiapoptotic activity. Overall, our data suggest that IMD, through its production by endothelial cells, may contribute to the regulation of vascular function in man. IMD, because it has marked responses in vivo [21] and in vitro as shown here, may be a potential marker of cardiovascular stress. IMD may possess a role in cell protection in human

endothelial cells and modulate vascular behaviour in a manner complementary to other peptides such as ADM.

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References

- Takei Y, Hyodo S, Katafuchi T, Minamino N: Novel fish-derived adrenomedullin in mammals: structure and possible function. *Peptides* 2004;25:1643-56.
- Roh J, Chang CL, Bhalla A, Klein C, Hsu SY: Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem* 2004;279:7264-7274.
- Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H, Miyano S: Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. *FEBS Lett* 2004;556:53-58.
- Takahashi K, Kikuchi K, Maruyama Y, Urabe T, Nakajima K, Sasano H, Imai Y, Murakami O, Totsune K: Immunocytochemical localization of adrenomedullin 2/intermedin-like immunoreactivity in human hypothalamus, heart and kidney. *Peptides* 2006;27:1383-1389.
- Fujisawa Y, Nagai Y, Miyatake A, Miura K, Shokoji T, Nishiyama A, Kimura S, Abe Y: Roles of adrenomedullin 2 in regulating the cardiovascular and sympathetic nervous systems in conscious rats. *Am J Physiol Heart Circul Physiol* 2006;290:H1120-H1127.
- Charles CJ, Rademaker MT, Richards AM: Hemodynamic, hormonal, and renal actions of adrenomedullin-2 in normal conscious sheep. *Endocrinology* 2006;147:1871-1877.
- Yang JH, Pan CS, Jia YX, Zhang J, Zhao J, Pang YZ, Yang J, Tang CS, Qi YF: Intermedin1-53 activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. *Biochem Biophys Res Comm* 2006;341:567-572.
- Morimoto R, Satoh F, Murakami O, Totsune K, Suzuki T, Sasano H, Ito S, Takahashi K: Expression of adrenomedullin2/intermedin in human brain, heart, and kidney. *Peptides* 2007;28:1095-1103.
- Kindt F, Wiegand S, Loser C, Nilles M, Niemeier V, Hsu SY, Steinhoff M, Kummer W, Gieler U, Haberberger RV: Intermedin: a skin peptide that is downregulated in atopic dermatitis. *J Invest Dermatol* 2007;127:605-613.
- Kato J, Tsuruda T, Kita T, Kitamura K, Eto T: Adrenomedullin: a protective factor for blood vessels. *Arterioscler Thromb Vasc Biol* 2005;25:2480-2487.
- Zhao Y, Hague S, Manek S, Zhang L, Bicknell R, Rees MC: PCR display identifies tamoxifen induction of the novel angiogenic factor adrenomedullin by a non estrogenic mechanism in the human endometrium. *Oncogene* 1998;16:409-415.
- Kato H, Shichiri M, Marumo F, Hirata Y: Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* 1997;138:2615-2620.
- Chun TH, Itoh H, Ogawa Y, Tamura N, Takaya K, Igaki T, Yamashita J, Doi K, Inoue M, Masatsugu K, Korenaga R, Ando J, Nakao K: Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. *Hypertension* 1997;29:1296-1302.
- Saito T, Itoh H, Chun TH, Fukunaga Y, Yamashita J, Doi K, Tanaka T, Inoue M, Masatsugu K, Sawada N, Sakaguchi S, Arai H, Mukoyama M, Tojo K, Hosoya T, Nakao K: Coordinate regulation of endothelin and adrenomedullin secretion by oxidative stress in endothelial cells. *Am J Physiol Heart Circul Physiol* 2001;281:H1364-H1371.
- Hayashi M, Shimosawa T, Fujita T: Hyperglycemia increases vascular adrenomedullin expression. *Biochem Biophys Res Comm* 1999;258:453-456.

- 16 Autelitano DJ, Tang F, Little PJ: Rapid regulation of adrenomedullin in metabolically compromised vascular smooth muscle cells. *J Hypertens* 1999;17:373-379.
- 17 Kumar S, Kasseckert S, Kostin S, Abdallah Y, Piper HM, Steinhoff G, Reusch HP, Ladilov Y: Importance of bicarbonate transport for ischaemia-induced apoptosis of coronary endothelial cells. *J Cell Mol Medicine* 2007;11:798-809.
- 18 Kunsch C, Medford RM: Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 1999;85:753-766.
- 19 Sueur S, Pesant M, Rochette L, Connat JL: Antiapoptotic effect of calcitonin gene-related peptide on oxidative stress-induced injury in H9c2 cardiomyocytes via the RAMP1/CRLR complex. *J Mol Cell Cardiol* 2005;39:955-963.
- 20 Bell D, McDermott BJ: Intermedin (adrenomedullin-2): a novel counter-regulatory peptide in the cardiovascular and renal systems. *Brit J Pharmacol* 2008;153:S247-S262.
- 21 Bell D, Zhao Y, McCoy FP, Devine AB, McDermott BJ: Differential effects of an anti-oxidant intervention on cardiomyocyte expression of adrenomedullin and intermedin and their receptor components in chronic nitric oxide deficiency. *Cell Physiol Biochem* 2007;20:269-82.
- 22 Minamino N, Kikumoto K, Isumi Y: Regulation of adrenomedullin expression and release. *Microsc Res Tech* 2002;57:28-39.
- 23 Pearson LJ, Rait C, Nicholls MG, Yandle TG, Evans JJ: Regulation of adrenomedullin release from human endothelial cells by sex steroids and angiotensin-II. *J Endocrinol* 2006;191:179-188.
- 24 Evans JJ, Youssef AH, Yandle TG, Lewis LK, Nicholls MG: Effects of endothelin-1 on release of adrenomedullin and C-type natriuretic peptide from individual human vascular endothelial cells. *J Endocrinol* 2002;175:225-232.
- 25 Miller MJ, Martinez A, Unsworth EJ, Thiele CJ, Moody TW, Elsasser T, Cuttitta F: Adrenomedullin expression in human tumor cell lines. Its potential role as an autocrine growth factor. *J Biol Chem* 1996;271:23345-51.
- 26 Sipos H, Torocsik B, Tretter L, Adam-Vizi V: Impaired regulation of pH homeostasis by oxidative stress in rat brain capillary endothelial cells. *Cell Mol Neurobiol* 2005;25:141-151.
- 27 Isumi Y, Shoji H, Sugo S, Tochimoto T, Yoshioka M, Kangawa K, Matsuo H, Minamino N: Regulation of adrenomedullin production in rat endothelial cells. *Endocrinology* 1998;139:838-846.
- 28 Li AE, Ito H, Rovira, II, Kim KS, Takeda K, Yu ZY, Ferrans VJ, Finkel T: A role for reactive oxygen species in endothelial cell anoikis. *Circ Res* 1999;85:304-310.
- 29 Kel AE, Gossling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, Wingender E: MATCH: A tool for searching transcription factor binding sites in DNA sequences. *Nucleic Acids Res* 2003;31:3576-3579.
- 30 Amati B, Littlewood TD, Evan GI, Land H: The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *Embo J* 1993;12:5083-5087.
- 31 Juin P, Hueber AO, Littlewood T, Evan G: c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. *Genes Dev* 1999;13:1367-1381.
- 32 Shichiri M, Kato H, Doi M, Marumo F, Hirata Y: Induction of max by adrenomedullin and calcitonin gene-related peptide antagonizes endothelial apoptosis. *Mol Endocrinol* 1999;13:1353-63.
- 33 Ozawa N, Shichiri M, Fukai N, Yoshimoto T, Hirata Y: Regulation of adrenomedullin gene transcription and degradation by the c-myc gene. *Endocrinology* 2004;145:4244-4250.
- 34 Cai H: Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. *Cardiovasc Res* 2005;68:26-36.
- 35 Sata M, Kakoki M, Nagata D, Nishimatsu H, Suzuki E, Aoyagi T, Sugiura S, Kojima H, Nagano T, Kangawa K, Matsuo H, Omata M, Nagai R, Hirata Y: Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. *Hypertension* 2000;36:83-8.
- 36 Chen L, Kis B, Hashimoto H, Busija DW, Takei Y, Yamashita H, Ueta Y: Adrenomedullin 2 protects rat cerebral endothelial cells from oxidative damage in vitro. *Brain Res* 2006;1086:42-9.
- 37 Tesfamariam B, DeFelice AF: Endothelial injury in the initiation and progression of vascular disorders. *Vasc Pharmacol* 2007;46:229-37.
- 38 Hagiwara M, Bledsoe G, Yang ZR, Smith RS, Chao L, Chao J: Intermedin Ameliorates Vascular and Renal Injury by Inhibition of Oxidative Stress. *Am J Physiol Renal Physiol* 2008;295:F1735-F1743.
- 39 Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi T, Nakao K: Adrenomedullin promotes proliferation and migration of cultured endothelial cells. *Hypertens Res* 2003;26 Suppl:S93-S98.
- 40 Bailey RJ, Hay DL: Pharmacology of the human CGRP1 receptor in Cos 7 cells. *Peptides* 2006;27:1367-1375.
- 41 Owji AA, Chabot JG, Dumont Y, Quirion R: Adrenomedullin-2/intermedin induces cAMP accumulation in dissociated rat spinal cord cells: evidence for the existence of a distinct class of sites of action. *J Mol Neurosci* 2008;35:355-361.
- 42 Taylor MM, Bagley SL, Samson WK: Intermedin/Adrenomedullin-2 inhibits growth hormone release from cultured, primary anterior pituitary cells. *Endocrinology* 2006;147:859-864.
- 43 Hay DL: What makes a CGRP2 receptor? *Clin Exp Pharmacol Physiol* 2007;34:963-971.