Urocortin induces interleukin-6 release from rat cardiomyocytes through p38 MAP kinase, ERK and NF-κB activation

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Abstract

CRH and its structurally related peptide urocortin (Ucn) are released under stress. Ucn is a potent agonist for CRH-receptor 2 (CRH-R2), which is strongly expressed in rodent heart. Stress induces Ucn mRNA expression in the heart, where it may be cardioprotective. However, increasing evidence indicates that Ucn may also have pro-inflammatory actions. Here, we show that neonatal rat cardiomyocytes express CRH-R2 by western blot analysis and Ucn induces interleukin-6 (IL-6) release in a time- and dose-dependent fashion. Ucn stimulates activation of ERK and p38 MAP kinases, while both MEK1 and p38 inhibitor block Ucn-induced IL-6 release. Ucn also activates nuclear factor kappa B (NF-κB) and a NF-κB inhibitor blocks Ucn-induced IL-6 release. Finally, the CRH-R antagonists x-helical (9–41) CRH and astressin-2B completely inhibit Ucn-induced IL-6 release, as well as activation of ERK, p38, and NF-κB. These findings indicate that Ucn induces IL-6 synthesis and release from neonatal rat cardiomyocytes. Our findings suggest that even though Ucn may confirm some protection on cardiomyocyte survival, it can also release IL-6, which is an independent risk factor for acute coronary syndrome. The precise role of cardiac Ucn in vivo remains to be elucidated.

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Introduction

The stress response is regulated by CRH, which activates the hypothalamic–pituitary–adrenal (HPA) axis typically leading to anti-inflammatory actions (Chrousos 1995). There are two main types of the G protein-coupled CRH receptors, CRH-receptor 1 (CRH-R1), and CRH-R2, which mediate the effects of the CRH family of structurally related peptides that includes CRH, urotensin, sauvagine, and the urocortins (Ucn). CRH-R2 has three different spliced forms, α, β, and γ, of which CRH-R2α is mainly in the CNS, while CRH-R2β is predominantly found in the periphery, especially in the heart (Nishikimi et al. 2000, Kimura et al. 2002) including cardiomyocytes (Okosi et al. 1998). Ucn mRNA is also expressed in the heart (Nishikimi et al. 2000). Species differences have been reported in the distribution of CRH-R2 in the cardiovascular system, showing 10× higher expression in mice than rats or humans cardiac muscle (Wasser et al. 2006).

Ucn has been generally considered to be cardioprotective, especially in ischemia-reperfusion (IR) injury (Bale et al. 2003, Latchman 2003, Townsend et al. 2007), but is also increased (Nishikimi et al. 2000, Ikeda et al. 2003) with parallel decrease in cardiac CRH-R2β expression in cardiac hypertrophy (Nishikimi et al. 2000, Ikeda et al. 2003). On the other hand, acute stress, CRH, and Ucn have been implicated in the pathophysiology of some neuroinflammatory disorders (Chrousos 1995, Karalis et al. 1997, Theoharides & Cochrane 2004) and myocardial ischemia (MI; Jiang et al. 1996, Krantz et al. 2000). Acute stress elevates plasma interleukin-6 (IL-6) levels in rodents (Ando et al. 1998, Nukina et al. 2001) and such levels are higher in apolipoprotein E (Apo E) knockout mice that develop atherosclerosis (Huang et al. 2003).

Local inflammation is now recognized as a key component of coronary artery disease (CAD; Packard & Libby 2008). The proinflammatory cytokine IL-6 is...
thought to contribute to the development of CAD (Huber et al. 1999), cardiomyopathy, congestive heart failure (Deliargyris et al. 2000) and MI (Miyao et al. 1993). IL-6 is a major inducer of C reactive protein (CRP) and both are elevated in and are associated with increased risk of CAD. The Health ABC study showed that plasma IL-6 levels had a stronger association with CAD than CRP (Cesari et al. 2003), while the PRIME study showed that only IL-6 remained significantly associated with MI (Luc et al. 2003). In another study, the incidence of future acute coronary events and mortality of patients with stable CAD or healed MI was strongly correlated with serum IL-6 levels over a 6-year observation (Fisman et al. 2006). In patients with acute coronary syndrome (ACS), plasma IL-6 was increased and appeared to derive primarily from a cardiac source (Deliargyris et al. 2000), but the exact cell type remains known. Cardiomyocytes are capable of releasing IL-6 in response to hypoxia and to cytokines (Yamauchi-Takahara et al. 1995, Coste et al. 2001).

Here, we show for the first time that neonatal rat cardiomyocytes express CRH-R2 protein and secrete IL-6 in response to Ucn through the activation of CRH-R2 and subsequently MAP kinases and nuclear factor kappa B (NF-kB).

Materials and methods

Culture of neonatal rat cardiomyocytes

This protocol was approved by the Institutional Animal Care Committee. Ventricular cardiomyocytes were isolated from the hearts of 1–3-day old neonatal male Sprague/Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA) using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ, USA). Cardiomyocytes were enriched by preplating for 60 min to allow the non-myocytes to adhere to the plate. Non-adherent cardiomyocytes were then removed by aspiration and non-myocytes to adhere to the plate. Non-adherent cardiomyocytes were then removed by aspiration and plated in DMEM/F-12 (Gibco) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA), 10% horse serum (Sigma), 1% penicillin/streptomycin (Gibco), 20 μM vitamin B12 (Sigma) at 37°C in 95% air and 5% CO2. For the first 3 days of culture, 0.1 mM 5-bromo-2′-deoxyuridine (BrdU, Sigma) was added to the medium to prevent proliferation of non-myocytes. The culture medium was changed 40–48 h following seeding, to serum deprived medium: DMEM/F-12 supplemented with 1X insulin-transferrin-selenium (Gibco), 0.5% penicillin/streptomycin, 1% fetal bovine serum and 20 μM vitamin B12; cardiomyocytes remained in this medium for 24 h before further experiments. All cells in culture were seen beating spontaneously, indicative of cardiomyocytes. Ucn is expressed in cells of the immune system (Karalis et al. 1997) including mast cells (Kempuraj et al. 2004).

We stained the cardiomyocytes with 1% toluidine blue solution (pH <2) for mast cells and with Giemsa stain for lymphocytes in the final cardiomyocyte preparation. Electron microscopy confirmed the absence of any fibroblasts or endothelocyte cells (data not shown).

IL-6 measurements

Time-course and dose-response studies of Ucn-stimulated IL-6 release from cardiomyocytes were conducted. Cells were either treated with 100 nM Ucn for different periods of time (0–72 h), or treated with different concentrations of Ucn (0–10 μM) for 48 h; following stimulation, the culture medium was collected and was assayed for IL-6 using the quantitative sandwich enzyme immunoassay technique (R&D).

Cyclic AMP assays

Cardiomyocytes were incubated in serum-free culture medium containing 100 nM Ucn for the designated times and cAMP was measured with an ELISA kit (R&D) as previously described (Heldwein et al. 1996).

Western blot analysis

For the detection of CRH-R2, heart tissue and isolated cardiomyocytes were disrupted in ice-cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.02% sodium azide, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml PMSF, and 1 μg/ml aprotinin) and were then centrifuged at 15 000 g for 15 min at 4°C. For the detection of phosphorylation of MAP kinases, cardiomyocytes were exposed to 100 nM Ucn in the presence or absence of inhibitors for the indicated time, after which the cells were lysed in lysis buffer, vortexed, and centrifuged at 15 000 g for 15 min at 4°C. Aliquots of tissue and cell lysates were kept for protein estimation (Bio-Rad). An equal amount of protein from each sample was mixed with an equal volume of 3X SDS sample buffer. The samples were boiled for 3 min and proteins were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, subsequently transferred onto the PVDF membrane (Immobilon-P) by the semi-dry method (BioRad). The membrane was then blocked with 5% dry milk in PBS containing 0.05% Tween-20 (PBS-T) and probed for 2 h at room temperature with two different rabbit polyclonal anti-CRH-R2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and (Imgenex, San Diego, CA, USA) or an antibody that detects phospho-ERK and phospho-p38 (Cell Signaling Danvers, MA, USA). For the detection of total ERK
and p38, the membrane was probed for 1 h at room temperature with either an anti-ERK1 antibody or an anti-p38 antibody. The membrane was washed in PBS-T and incubated with a HRP-conjugated secondary antibody (Santa Cruz) for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

Electromobility gel shift assays

The nuclear extract of cardiomyocytes was collected as previously described (Li et al. 2001). The consensus NF-κB oligonucleotide (AGTTGAGGGGACTTTCC-CAGGC, Santa Cruz) was radiolabeled by mixing 50 ng oligonucleotide, 70 μCi [γ-32P] ATP, 1 μl T4 polynucleotide kinase, 1.5 μl 10×T4 polynucleotide buffer and double-distilled water in a 15 μl reaction volume. The nuclear extract protein (10 μg) was then mixed with 0.5 ng [γ-32P] ATP-labeled NF-κB oligonucleotide, 20 μg BSA, 2 μg poly dC, 2 μl Buffer D+ (20 mM Hepes pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40), 4 μl Buffer F (20% FICOLL 400, 100 mM Hepes pH 7.9, 300 mM KCl), and DTT (2 mM) in 20 μl total volume at room temperature for 20 min. At the end of the reaction, the mixture was loaded on a non-denaturing 4% polyacrylamide gel using a running buffer containing 50 mM Tris pH 7.5, 0.38 M glycine, and 2 mM EDTA, after which the gel was dried and exposed to Kodak X-ray film for autoradiography at −80°C.

Drug pretreatment

Cardiomyocytes were pretreated with the p38 MAP kinase inhibitor SB203580 (10 μM) or the MEK1 inhibitor PD98059 (20 μM) 1 h before Ucn treatment. The NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was added at 50 μM to the culture medium 2 h before Ucn treatment. The peptide CRH-R antagonists α-helical (9–41) CRH or astressin-2B (10 μM) were added 1 h before Ucn treatment. The optimal concentrations and the duration of pretreatment for all the inhibitors used were predetermined by time- and dose-response experiments (not shown). All concentrations of the different inhibitors used in these experiments have been previously shown not to be toxic to cardiomyocytes; moreover, the viability of the cardiomyocytes was intact, as they were still beating and Trypan blue exclusion was minimal at 48 h.

Statistical analysis

Each experiment was performed at least four times unless stated otherwise. The differences between unstimulated and stimulated cells were compared using both the two-tailed Student’s t-test and the non-parametric Mann–Whitney U-test; comparison between different treatment groups was performed by ANOVA using one-way ANOVA. Results are presented as mean±s.e.m. For all analyses, P<0.05 was considered to indicate statistical significance.

Results

Time- and dose-dependent increase of IL-6 release by urocortin

Ucn increased IL-6 release from isolated cardiomyocytes in a time- and dose-dependent fashion. Significant increase of IL-6 release was observed as early as 4 h of Ucn (100 nM) exposure. Ucn produced 15.5±0.5 pg/ml of IL-6 as compared with 7.1±1.4 pg/ml from unstimulated cells (Fig. 1A, n=6, P<0.05). The Ucn-induced IL-6 release increased with time and maximal release was observed at 48 h of stimulation when Ucn treated cells produced 120.2±9.5 pg/ml as
compared with 59.1 ± 7.2 pg/ml of IL-6 from untreated cells (Fig. 1A, n=6, P<0.05). Unstimulated cells also released more IL-6 over time, but the difference between them and the treatment group remained significant at every time point (Fig. 1A). Ucn-induced IL-6 release from cardiomyocytes was also dose-dependent. When cells were treated with different concentrations of Ucn for 48 h, 10 nM was the lowest concentration of Ucn that could stimulate significant IL-6 increase of 83.5 ± 5.5 pg/ml versus 59.1 ± 7.2 pg/ml in controls (Fig. 1B, n=6, P<0.05). Maximal IL-6 release of 148.3 ± 8.1 pg/ml was induced by 1 μM of Ucn (Fig. 1B, n=6, P<0.05); higher Ucn concentrations (10 μM) did not increase IL-6 release any further (152.0 ± 6.1 pg/ml, Fig. 1B, n=6).

### Presence of CRH-R2 in cardiomyocytes

Western blot analysis using anti-CRH-R2 antibodies from two different sources demonstrated the presence of a strong immunoreactive band of about 49 kDa in mouse and rat heart, as well as in neonatal rat cardiomyocytes (positive controls included rat cortex and cerebellum; Fig. 2A–C). Use of a blocking peptide for CRH-R2 abolished immunoreactivity, while one for CRH-R1 had no effect (data not shown). There was no CRH-R1 expression noted (data not shown). In order to investigate whether the CRH-R2 on cardiomyocytes is functional, cellular cAMP levels were measured following Ucn stimulation, since CRH and related peptides typically bind to CRH-R and activate adenylate cyclase. Production of cAMP increased significantly by 3.4 ± 0.2-fold above basal levels after 2 min of stimulation with 100 nM Ucn, reaching a maximum of 6.9 ± 0.2-fold above basal level (n=6, P<0.05) at 5 min, and then decreased slowly over time, but still remained 5.3 ± 0.1-fold above basal values at 20 min (results not shown).

### Urocortin activates ERK and p38 MAP kinases

In order to study the possible mechanism(s) by which Ucn stimulates IL-6 release, we investigated the effect of Ucn on the phosphorylation of MAP kinases, including ERK and p38. Cardiomyocytes responded to 100 nM Ucn with the activation of both ERK and p38. Phosphorylation of ERK (p44/42) was observed at 2 min and was maximized at 5 min after which it decreased over time, but remained above basal level up to 1 h (Fig. 3A).

The peptide CRH-R inhibitor astressin completely reversed the effect of Ucn (100 nM for 15 min) on ERK activation (Fig. 3A). Pretreatment with PD98059 (200 nM for 1 h), the specific inhibitor of MEK1, which is the upstream stimulator of ERK, also completely inhibited the phosphorylation of ERK. The specific inhibitor of p38 MAP kinase SB203580 (100 nM) had no apparent effect on ERK activation (Fig. 3A). There was no difference in total ERK between control samples, the positive control stimulated by IL-1β and Ucn (Fig. 3A, lower panel).

Phosphorylation of p38 MAP kinase occurred later than that of ERK (Fig. 3A and B); there was a moderate increase of p38 phosphorylation observed at 15 min of Ucn exposure (Fig. 3B). Both CRH-R antagonists, α-helical (9–41) CRH and astressin completely...
Urocortin stimulates cardiomyocyte IL-6

Discussion
Here, we show for the first time that Ucn stimulates IL-6 release from neonatal rat cardiomyocytes. CRH-R2 appears to be involved, since it is identified in cardiac tissue and in isolated cardiomyocytes by immunoblotting, and the release of IL-6 is blocked by astressin-2B. CRH-R2 was detected by antibodies from two different sources, using rat cortex and cerebellum as positive controls. The same Santa-Cruz antibody used here was previously reported to be specific (Bishop et al. 2006), and also identified a band around 49 kDa. Moreover, the western blot with the second anti-CRH-R2 antibody from Imgenex also generated a band around 49 kDa, as shown in the product specification. A similar molecular weight band is also recognized by more anti-CRH-R2 antibodies (data not shown) by Novus Biologicals (Littleton, CO, USA; www.novusbio.com/data_sheet/index/NB100-56485). Some authors have reported the molecular weight for the full length CRH-R2 to be about 70 kDa in the pituitary, but lower in

Urocortin increases IL-6 release by a CRH-R2-induced MAP kinase- and NF-κB-dependent mechanism

In order to determine whether Ucn-induced IL-6 release from cardiomyocytes involves MAP kinases or NF-κB activation, cardiomyocytes were pretreated for 1 h before Ucn (100 nM) stimulation with either the specific inhibitor of p38 MAP kinase SB203580 (10 μM) or the MEK1 inhibitor PD98059 (20 μM). Otherwise, cardiomyocytes were pretreated with the NF-κB inhibitor PDTC (50 μM) for 2 h before Ucn addition. As shown in Fig. 5A, both SB203580 and PD98059 completely inhibited Ucn-induced IL-6 release, indicating that both p38 and ERK are involved in Ucn-stimulated IL-6 release from cardiomyocytes. PDTC (50 μM for 1 h) also completely inhibited IL-6 release stimulated by Ucn, further indicating the involvement of NF-κB. Both CRH-R2 to be about 70 kDa in the pituitary, but lower in

Urocortin activates NF-κB

There was moderate activation of NF-κB after Ucn treatment (100 nM, 15 min to 6 h duration), as shown by gel-shift assay (Fig. 4A). Both α-helical CRH (9–41) and SB203580 completely inhibited Ucn (100 nM for 15 min)-induced NF-κB activation (Fig. 4A), suggesting that the activation of NF-κB in cardiomyocytes is mediated by CRH-R and is dependent on p38 phosphorylation. Pretreatment with PD98059 (20 μM for 1 h) unexpectedly did not have any effect on NF-κB activation. Supershift assay using an antibody against the p50 subunit of NF-κB confirmed that the Ucn-stimulated increase in binding resulted from NF-κB activity in the nuclear extracts of Ucn-treated cells (Fig. 4B).
the brain (Grigoriadis & De Souza 1988); the 49 kDa band may represent a CRH-R2 form that may be truncated, with less glycosylation, or a tissue specific variant.

Ucn and CRH-R2α mRNA expression has been detected in cardiac tissue with CRH-R2β predominantly expressed in the left ventricle (Kimura et al. 2002). CRH-R2 protein expression was shown by autoradiography using a specific radiolabeled anti-CRH-R2 ligand to be very high in rodent heart, with very little CRH-R1 detected (Waser et al. 2006). CRH-R2β mRNA expression was depressed in left ventricular hypertrophy, possibly through downregulation by Ucn (Nishikimi et al. 2000). Restraint stress upregulated Ucn mRNA, but decreased CRH-R2β mRNA levels in the rat heart and aorta (Pourmaji et al. 2003). CRH-R2 mRNA expression was also downregulated by IL-1 and TNF in the mouse heart (Coste et al. 2001), possibly acting to limit the inotropic and chronotropic effects of Ucn.

Ucn II was shown to stimulate rat neonatal cardiomyocytes to release atrial natriuretic peptide in a CRH-R2 and cAMP-dependant manner (Ikeda et al. 2005). Ucn mediates stress-induced IL-6 release in vivo and administration of Ucn causes elevation of plasma IL-6 in rats (Ando et al. 1998). Ucn also stimulates IL-6 secretion from human peripheral mononuclear cells in vitro (Kohno et al. 2001), as well as an increase in IL-6 mRNA levels through CRH-R2 in rat aortic smooth muscle cells (Kageyama et al. 2007).

Our study demonstrates that IL-6 release by Ucn involves p38 MAP kinase, ERK and NF-κB activation. Our results are consistent with a previous study that showed p38 MAP kinase activation by Ucn, but not CRH, in cultured human pregnant myometrial cells (Grammatopoulos et al. 2000), as well as NF-κB involvement in TNF-induced IL-6 expression and release from cardiomyocytes (Craig et al. 2000) and upregulation of inflammatory IL-6 expression (TNF-α, IL-1 and IL-6) in cardiomyocyte hypertrophy (Purcell et al. 2001). Surprisingly, our results show that while Ucn-induced IL-6 release requires NF-κB activation, Ucn-induced NF-κB activation does not appear to require ERK. This discrepancy may indicate that ERK1/2 contributes to Ucn stimulation of IL-6 production independent of NF-κB.

**Figure 4** Effect of Ucn on NF-κB in cardiomyocytes. (A) NF-κB activation demonstrated by binding of NF-κB to the radiolabeled oligonucleotide shown by EMSA and effects of α-helical (9–41) CRH, SB203580 (10 μM) and PD98059 (20 μM). (B) Supershift assay using antibody against the p50 subunit of NF-κB confirmed that the Ucn-stimulated increase in binding resulted from NF-κB activity in the nuclear extracts of Ucn-treated cells (representative gel from n=5; *P<0.05).

**Figure 5** Ucn increases IL-6 release in cardiomyocytes through CRHR MAP kinase and NF-κB. (A) Pretreatment with SB203580 at 10 μM, PD98059 at 20 μM for 1 h before Ucn stimulation (100 nM, 48 h) or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) at 50 μM 2 h before Ucn stimulation (100 nM, 48 h) (n=6; *P<0.05 versus control; ^P<0.05 versus Ucn 100 nM). (B) Pretreatment with α-helical (9–41) CRH or astressin-2B at 10 μM for 1 h before Ucn stimulation (100 nM, 48 h) (n=5; *P<0.05 versus control; ^P<0.05 versus Ucn 100 nM).
The role of Ucn in the heart is far from clear. Ucn protects the rat heart from IR injury in vitro and in vivo through upregulation of the p42/p44 MAPK pathway (Schulman et al. 2003). Ucn-II and Ucn-III are also cardioprotective against IR injury (Brar et al. 2004, Woodcock 2004). Stimulation of CRH-R2β by Ucn-II and III resulted in cAMP-independent ERK1/2 phosphorylation during IR and reduced infarct size (Brar et al. 2004). Both Ucn (Brar et al. 2000) and IL-6 (Craig et al. 2000) may protect neonatal rat cardiomyocytes from apoptosis induced by hypoxia. However, while i.v. administration of Ucn-II had inotropic and chronotropic effects in mice and humans, it decreased blood pressure, effects absent in CRH-R2 knockout mice (Bale et al. 2004, Davis et al. 2007). Ucn-II also decreased mean arterial blood pressure in the rat, which was blocked by CRH-R2 antagonist antisauvagine without any effect on normal cardiac function (Mackay et al. 2008). Ucn is released before and independently of arteriovenous pressure in the rat, which was blocked by the CRH-R2 antagonist anti-sauvagine without any effect on normal cardiac function (Mackay et al. 2003). The importance of inflammation including IL-6 and its stimulation of CRP production in atherosclerosis was reviewed recently (Packard & Libby 2008).

Cardiomyocyte-derived IL-6 in response to Ucn may even have dual and opposing actions. It might originally lead to inhibition of apoptosis, but eventually to hypertrophy alone or following angiotensin II (Sano et al. 2000). In macrophages, both CRH-R1 and CRH-R2 agonists have an early anti-inflammatory, but delayed pro-inflammatory effect on TNF-α release (Tsatsanis et al. 2007). The particular effects of Ucn may depend on the stage of maturation of the cardiomyocytes and/or activation of specific CRH-R2 isoforms, documented in keratinocytes (Pisarchik & Slominski 2004) and mast cells (Cao et al. 2005). For instance, a soluble CRH-R2ζ isoform was shown to neutralize the effect of CRH-R agonists (Chen et al. 2005), while Ucn mRNA antisense transcripts have been identified in rat tissue (Shi et al. 2000).

Acute stress is implicated in cardiovascular pathology (Theoharides & Cochrane 2004), especially in eliciting MI in patients with CAD (Jiang et al. 1996). Acute stress elevates plasma IL-6 levels in rodents (Ando et al. 1998, Nukina et al. 2001) and such levels are higher in ApoE knockout mice that develop atherosclerosis (Huang et al. 2003). IL-6 plays a crucial role in MI (Miyao et al. 1993), CAD (Huber et al. 1999), cardiac hypertrophy (Sano et al. 2000), and congestive heart failure (Deliargyris et al. 2000). IL-6 expression is also increased in coronary arteries of aged rats that are more prone to atherosclerosis and CAD (Cesari et al. 2003). The importance of inflammation including IL-6 and its stimulation of CRP production in atherosclerosis was reviewed recently (Packard & Libby 2008).

Elevated intracoronary levels of IL-6 (Deliargyris et al. 2000) shown in patients with acute CAD may also be involved in coronary hypersensitivity leading to Kounis syndrome (Kounis et al. 2007). Consequently, any suggestion that Ucn peptides could be used therapeutically for heart conditions may be too premature.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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