CB1 receptor mediates the effects of glucocorticoids on AMPK activity in the hypothalamus

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Abstract

AMP-activated protein kinase (AMPK), a regulator of cellular and systemic energy homeostasis, can be influenced by several hormones. Tissue-specific alteration of AMPK activity by glucocorticoids may explain the increase in appetite, the accumulation of lipids in adipose tissues, and the detrimental cardiac effects of Cushing’s syndrome. Endocannabinoids are known to mediate the effects of various hormones and to influence AMPK activity. Cannabinoids have central orexigenic and direct peripheral metabolic effects via the cannabinoid receptor type 1 (CB1). In our preliminary experiments, WT mice received implants of a corticosterone-containing pellet to establish a mouse model of Cushing’s syndrome. Subsequently, WT and Cb1 (Cnr1)-knockout (CB1-KO) littermates were treated with corticosterone and AMPK activity in the hypothalamus, various adipose tissues, liver and cardiac tissue was measured. Corticosterone-treated CB1-KO mice showed a lack of weight gain and of increase in hypothalamic and hepatic AMPK activity. In adipose tissues, baseline AMPK activity was higher in CB1-KO mice, but a glucocorticoid-induced drop was observed, similar to that observed in WT mice. Cardiac AMPK levels were reduced in CB1-KO mice, but while WT mice showed significantly reduced AMPK activity following glucocorticoid treatment, CB1-KO mice showed a paradoxical increase. Our findings indicate the importance of the CB1 receptor in the central orexigenic effect of glucocorticoid-induced activation of hypothalamic AMPK activity. In the periphery adipose tissues, changes may occur independently of the CB1 receptor, but the receptor appears to alter the responsiveness of the liver and myocardial tissues to glucocorticoids. In conclusion, our data suggest that an intact cannabinoid pathway is required for the full metabolic effects of chronic glucocorticoid excess.
Introduction

Cushing’s syndrome results from chronic exposure to high doses of glucocorticoids. Endogenous Cushing’s syndrome is rare and may be caused by an adrenocorticotropin (ACTH)-secreting pituitary adenoma (Cushing’s disease) or a cortisol-secreting adrenal tumour or, more rarely, by ectopic ACTH or corticotrophin-releasing hormone (CRH) production. On the other hand, exogenous (iatrogenic) Cushing’s syndrome results from chronic exposure to high doses of glucocorticoids. Endogenous Cushing’s syndrome is associated with increased hypothalamic endocannabinoid content (Muisesan et al. 2003). Adverse effects on muscle (myopathy), bone (osteoporosis), mood and cognitive function also occur. These manifestations lead to increased cardiovascular risk, impaired quality of life and reduced life expectancy (Pivonello et al. 2005).

Many of the changes observed during glucocorticoid excess correspond to the metabolic steps regulated by AMP-activated protein kinase (AMPK). AMPK is a regulator of cellular and systemic energy homeostasis and acts as a sensor of energy status. AMPK may be activated through physiological and pathological mechanisms (Kahn et al. 2005). The inhibition of AMPK synthesis by pathological processes such as glucose deprivation, ischaemia, hypoxia, heat shock, metabolic poisons and oxidative stresses leads to AMPK activation. Physiologically, muscle contraction accelerates ATP consumption, thus activating AMPK. Once activated, AMPK switches off anabolic pathways, such as fatty acid, triglyceride and cholesterol synthesis, in favour of catabolic pathways, such as glycolysis and fatty acid oxidation. With the aim of restoring energy balance, ATP production is increased and energy-utilising pathways are inhibited. AMPK has acute effects through the direct phosphorylation of target enzymes and long-term effects through the regulation of gene and protein expression (Kola et al. 2006).

The activation of hypothalamic AMPK leads to an increase in appetite and that of myocardial AMPK is protective in myocardial ischaemia (Kola et al. 2006).

The activation of AMPK leads to reduced adipose tissue lipid stores with inhibition of lipogenesis and also to reduced lipolysis with reduction in free fatty acid release. Interestingly, AMPK activity is lower in the adipose tissues of patients who are insulin-resistant than in those of BMI-matched insulin-sensitive subjects (Gauthier et al. 2011), and in individuals treated with metformin, AMPK activation has been demonstrated (Boyle et al. 2011). The activation of AMPK in the liver leads to the inhibition of gluconeogenesis and fatty acid and cholesterol synthesis. AMPK mediates the action of several metabolic hormones. These hormones include leptin, adiponectin, insulin and ghrelin (Kola et al. 2006). We have shown previously that glucocorticoids have tissue-dependent regulatory effects on AMPK activity (Christ-Crain et al. 2008, Kola et al. 2008). AMPK has also been implicated in the mediation of the metabolic effects of cannabinoids (Kola et al. 2005).

Cannabinoids have central and peripheral metabolic effects via the cannabinoid receptor type 1 (CB1), which is widely expressed in the hypothalamus (Wittmann et al. 2007), but also found in peripheral tissues including adipose tissues, liver and the myocardium (Butler & Korbonits 2009). The activation of central hypothalamic CB1 receptors is associated with appetite stimulation (Cota et al. 2003). Cannabinoids have been reported to have cardioprotective roles in myocardial ischaemia (Underdown et al. 2005), and cannabinoid actions on the cardiovascular system have been widely interpreted as being mediated by CB1 receptors, although some of the reported cardiovascular effects of cannabinoids are mediated by non-CB1 receptors (Hiley 2009). In adipose tissues and liver, CB1 receptor activation promotes fat accumulation (Cota et al. 2003, Osei-Hyiaman et al. 2005, 2008).

It appears that the cannabinoid–CB1 system interacts with a number of hormonal systems and can mediate their effects (Di Marzo et al. 2001, Kola et al. 2008, Butler & Korbonits 2009, Turu et al. 2009). Glucocorticoids stimulate endocannabinoids in the hypothalamus (Di et al. 2003, 2005). We have shown previously glucocorticoid-induced changes in hypothalamic AMPK to be associated with increased hypothalamic endocannabinoid content (Christ-Crain et al. 2008). This suggests that the endogenous cannabinoid system may be required for the effects of glucocorticoids on hypothalamic AMPK activity and ultimately appetite. While glucocorticoids and cannabinoids have opposing effects on AMPK activity in the liver and myocardium, we have shown previously that both glucocorticoids and cannabinoids stimulate AMPK activity...
in the hypothalamus and inhibit it in adipose tissues (Kola et al. 2005, 2008, Christ-Crain et al. 2008). In the present study, we established a mouse model of Cushing’s syndrome and we further investigated the role of the CB1 receptor in the tissue-specific effects of glucocorticoids on AMPK activity by utilising Cb1 (Cnr1)-knockout (CB1-KO) mice.

Materials and methods

Animals

CB1-KO mice were generated at the Université Libre de Bruxelles (Ledent et al. 1999) and bred at the Institute of Experimental Medicine, Hungarian Academy of Sciences (Budapest). Adult male WT and CB1-KO mice were housed under standard environmental conditions (light between 0600 and 1800 h, temperature 22 ± 1 °C, and rodent chow and water provided with the animals allowed to feed and drink ad libitum). All experimental protocols were reviewed and approved by the local Animal Welfare Committee.

Initial experiments to establish a mouse model of Cushing’s syndrome using WT mice indicated that the 40 mg/mouse corticosterone dose, equivalent in terms of milligram corticosterone/body weight in the previously established rat model (Bell et al. 2000, Dallman et al. 2003) and used by us (Christ-Crain et al. 2008), was excessively high, leading to cachexia and fatal diabetes. With the aim of finding an adequate corticosterone dose to induce Cushing’s syndrome, preliminary experiments were carried out in WT mice treated with a surgically implanted s.c. pellet containing 40 mg cholesterol (placebo control) or a 40 mg pellet containing a mixture of cholesterol and corticosterone (corticosterone content 5, 10 or 20 mg; n = 5 for each group). The pellets containing varying amounts of corticosterone were prepared using commercially available moulds, as described previously (Bell et al. 2000). Animals were provided with a bottle of 0.5% NaCl and a second bottle containing 30% sucrose and allowed to drink ad libitum. Body weight and calorie (chow and sucrose) intake were recorded daily. Mice were killed 2 weeks after the insertion of pellets. Plasma samples were collected in the morning during killing and assayed for corticosterone, ACTH (Zelena et al. 2011) and glucose. The preliminary experiments revealed that 5 mg corticosterone was sufficient to induce Cushing’s syndrome. Therefore, WT and CB1-KO littermates were treated with a surgically implanted s.c. pellet containing 40 mg cholesterol (placebo control) or 35 mg cholesterol + 5 mg corticosterone (n = 5–6 for each group). Tissues (hypothalamus, visceral (mesenteric, perirenal and epididymal) and subcutaneous (inguinal) adipose tissues, liver and heart) were frozen on dry ice and stored at −80 °C.

Measurement of AMPK activity

The kinase assays for determining AMPK activity have been described previously (Hawley et al. 2003, Kola et al. 2005). Briefly, tissues were weighed and homogenised with a Precellys 24 machine using CK14 tubes containing ceramic beads (Stretton Scientific, Stretton, UK) at 6500 r.p.m. for three cycles of 20 s in a lysis buffer containing 50 mM Tris–HCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM PMSF and 5 μg/ml soybean trypsin inhibitor. Tissue protein content was determined using the BCA assay (Pierce, Rockford, IL, USA). AMPK was immunoprecipitated with an equal mixture of z1AMPK and z2AMPK antibodies, and AMPK activity was determined by the entity of 32P-incorporation into SAMS (amino acid sequence: HMRSAMSGLHLVKRR) (Pepceuticals Ltd., Nottingham, UK), a synthetic peptide substrate of AMPK.

Statistical analysis

Statistical analysis of data was performed on StatsDirect (Ian Buchan, Cambridge, UK). Data were analysed using the Kruskal–Wallis test followed by the Conover–Inman test for multiple group comparisons. Data are expressed as means ± S.E.M.s, n = 5–6 in each treatment group. To allow for comparison between experiments performed on different days and comparison between groups of different genetic backgrounds, AMPK assay results are presented as the percentage of controls, where control refers to WT mice treated with corticosterone (placebo) pellets. A P value < 0.05 was considered significant.

Results

Mouse model of Cushing’s syndrome

Hormonal parameters confirmed that glucocorticoid-treated WT mice had the expected profile of Cushing’s syndrome. WT mice treated with 5, 10 and 20 mg corticosterone showed significantly increased plasma corticosterone levels (P = 0.0006, P < 0.0001 and P < 0.001 respectively; Fig. 1A). Conversely, plasma ACTH levels were decreased with corticosterone treatment as expected (Fig. 1B). WT mice treated with low-dose (5 mg) corticosterone displayed significant weight gain (P = 0.0356 vs WT
control), while the other two groups were not significantly different from the control mice (Fig. 1C). Corticosterone-treated WT mice showed a tendency to increase calorie intake, but there were considerable variations and these were not significant (Fig. 1D). WT mice treated with the higher doses of corticosterone (10 and 20 mg) had significantly higher glucose levels and became diabetic (Fig. 1E). WT mice treated with low-dose corticosterone (5 mg) showed increased visceral (perirenal; P < 0.0053 vs WT control; Fig. 1F) and subcutaneous (inguinal; P < 0.0095 vs WT control; Fig. 1G) fat pad weight.

Since mice treated with the 5 mg corticosterone-containing pellet showed the characteristic metabolic changes of Cushing’s syndrome, this pellet was used in further experiments using CB1-KO mice. Glucocorticoid-treated (5 mg) CB1-KO mice showed no increase in calorie intake (P = 0.4724 vs CB1-KO control) and a trend to reduced body weight (P = 0.0514 vs CB1-KO control).

Hypothalamus

WT mice treated with a s.c. pellet containing 5 mg corticosterone showed significantly increased AMPK activity in the hypothalamus compared with WT mice treated with the cholesterol pellet (P = 0.0008 vs WT Chol; Fig. 2). In CB1-KO mice, this effect was lost: CB1-KO mice treated with 5 mg corticosterone did not show increased AMPK activity in the hypothalamus (P = 0.6079 vs CB1-KO Chol; Fig. 2). WT and CB1-KO mice treated with cholesterol (control) did not show any significant difference in hypothalamic AMPK activity (P = 0.3275; Fig. 2). However, glucocorticoid-treated CB1-KO mice
showed lower hypothalamic AMPK activity compared with their WT equivalents (P = 0.0034; Fig. 2). These results indicate the importance of the CB1 receptor in the mediation of the stimulatory effect of glucocorticoids on hypothalamic AMPK activity.

**Adipose tissues**

WT mice treated with the 5 mg corticosterone pellet showed significantly increased white adipose tissue depot weight (P = 0.0219 vs WT Chol). CB1-KO mice showed reduced baseline white adipose tissue depot and glucocorticoid-treated white adipose tissue depot weight when compared with their WT equivalents (P = 0.0185 vs WT Chol and P = 0.0048 vs WT 5 mg Cort; Fig. 3A).

Glucocorticoid-treated WT mice showed a significant reduction in visceral fat AMPK activity (P = 0.0006 vs WT Chol; Fig. 3B). Glucocorticoid-treated CB1-KO mice also showed a significant reduction in visceral fat AMPK activity when compared with their WT equivalents (Fig. 3B).

Similarly, glucocorticoid-treated WT mice showed a significant reduction in subcutaneous (inguinal) fat AMPK activity (P < 0.0001 vs WT Chol; Fig. 3C). Glucocorticoid-treated CB1-KO mice also showed a significant reduction in subcutaneous fat AMPK activity (P = 0.0013 vs CB1-KO Chol; Fig. 3C). Basal and glucocorticoid-treated CB1-KO groups showed significantly increased visceral fat AMPK activity when compared with their WT equivalents (Fig. 3C).
Liver

WT mice treated with 5 mg corticosterone showed a significant increase in hepatic AMPK activity (P<0.004 vs WT Chol; Fig. 4), similar to our rat model (Christ-Crain et al. 2008). CB1-KO mice showed significantly higher baseline liver AMPK activity when compared with their WT littermates (P<0.0356). However, there was no increase in hepatic AMPK activity after glucocorticoid treatment in CB1-KO mice (Fig. 4).

Heart

Glucocorticoid-treated WT mice showed a significant reduction in cardiac AMPK activity (P<0.016 vs WT control; Fig. 5A). CB1-KO mice showed significantly reduced baseline cardiac AMPK activity when compared with their WT equivalents (P=0.0056; Fig. 5A), and in contrast to WT mice, this reduced AMPK activity was significantly increased by glucocorticoid treatment (P<0.0075 vs CB1-KO control).

Discussion

We have suggested previously that many of the detrimental effects of Cushing’s syndrome are mediated, at least partially, by glucocorticoid-induced changes in AMPK activity in both humans and rats (Christ-Crain et al. 2008, Kola et al. 2008). In the present study, we showed that some of the detrimental effects of glucocorticoids are mediated by the endocannabinoid system.

Hypothalamic AMPK has been shown to play a vital role in the regulation of food intake (Minokoshi et al. 2004, Dzamko et al. 2010) and to be influenced by appetite-regulatory hormones such as leptin, ghrelin and cannabinoids (Andersson et al. 2004, Minokoshi et al. 2004, Kola et al. 2005). In this study, glucocorticoid-treated WT mice displayed significantly increased hypothalamic AMPK activity. Glucocorticoids stimulate hypothalamic AMPK, and data indicate that AMPK may mediate the orexigenic effect of glucocorticoids. Glucocorticoids have been shown to up-regulate the gene expression of orexigenic peptides (neuropeptide Y and agouti-related peptide) in rat arcuate nucleus via AMPK (Shimizu et al. 2008). Glucocorticoids can also stimulate endocannabinoids in the hypothalamus, as shown in hypothalamic cells in vitro (Di et al. 2003, 2005) and by increasing endocannabinoid content in vivo (Christ-Crain et al. 2008). Hypothalamic endocannabinoid levels have been shown to increase with fasting and to decrease after re-feeding (Kirkham et al. 2002) and to be involved in the mediation of the anorectic effect of leptin (Di Marzo et al. 2001) and of the orexigenic effect of ghrelin (Kola &
Korbonits 2009). In this study, we showed that CB1-KO mice are protected from the glucocorticoid-induced increase in hypothalamic AMPK activity.

In humans, the response to glucocorticoids is usually weight gain, but rapid development of high glucocorticoid levels, such as in ectopic ACTH-secreting neuroendocrine tumours, can result in significant catabolism and lack of weight gain. Reduction in weight gain is more often observed in rodents in response to glucocorticoids. In order to achieve the metabolic condition resembling human Cushing’s syndrome, glucocorticoid administration in the rodent Cushing’s syndrome model, originally set up by Dallman’s group, needs to be accompanied by high-dose carbohydrate intake (sucrose drink; Bell et al. 2000). This pronounced catabolic effect of glucocorticoids in rodent models is represented by the decreasing body and fat pad weight with increasing doses of glucocorticoid treatment, where the severe diabetes also contributes to the weight loss. Interestingly, a recent study has described a unique novel mechanism of human, but not rodent, visceral fat accumulation, and this could explain the difference in glucocorticoid sensitivity (Lindroos et al. 2013).

Hypercortisolaemia is associated with increased adipose tissue deposits in patients (Rockall et al. 2003a, Geer et al. 2010). Treatment of both WT and CB1-KO mice with corticosterone resulted in a significant reduction of adipose tissue AMPK activity, indicating that the CB1 receptor does not mediate the inhibitory effect of glucocorticoids on adipose tissue AMPK activity. However, it is interesting to note that baseline AMPK activity in the adipose tissues of CB1-KO mice is significantly higher than that in the adipose tissues of WT mice, highlighting the role of the CB1 receptor in the reduction of adipose tissue AMPK activity. Our AMPK assay results are concordant with the results obtained for fat depot weight. Corticosterone treatment is associated with decreased adipose tissue AMPK activity and increased adipose tissue weight. The interesting difference between the two genotypes is that CB1-KO mice show higher adipose tissue AMPK activity and lower adipose tissue weight.

Many of the lipid-accumulating effects of cannabinoids can be explained by their inhibitory effect on adipose tissue AMPK activity (Kola et al. 2005). Chronic blockade of CB1 receptors of SV40 immortalised murine white adipocytes with rimonabant results in significantly increased phosphorylation and activation of AMPK (Perwitz et al. 2010) and blockade of the CB1 receptor leads to a significant reduction of white adipose tissue weight (Jbilo et al. 2005). CB1 antagonism in diet-induced obese mice is associated with the normalisation of adipocyte metabolism (Jourdan et al. 2010). In this study, we showed that CB1-KO mice have significantly higher adipose tissue AMPK activity and significantly lower adipose tissue weight when compared with their WT equivalents. However, CB1-KO mice are not protected from the inhibitory effects of glucocorticoids on adipose tissue AMPK activity. Thus, in this study, we showed that the CB1 receptor does not mediate the inhibitory effect of glucocorticoids on adipose tissue AMPK activity.

Cushing’s syndrome is associated with increased hepatic fat accumulation (Rockall et al. 2003b); therefore, reduced hepatic AMPK activity could be expected in glucocorticoid-treated animals. However, in both rat and mouse studies, increased hepatic AMPK has been observed (Viana et al. 2006, Christ-Crain et al. 2008). Although the activation of AMPK suppresses nuclear accumulation of the lipogenic transcription factor SREBP-1c (SREBF1), resulting in reduced transcription of its target genes, acetyl-CoA carboxylase (Acc (Acaca)) and fatty acid synthase (Fas (Fasn)), and inhibition of aberrant lipogenesis in the liver (Li et al. 2011), it is important to note that liver metabolism is complex in high glucocorticoid states. The direct effect of glucocorticoids is combined with the effect of high glucose and high insulin levels, as well as increased net flux of fatty acids into the liver (Foretzi et al. 2005), leading to enhanced hepatic lipid oxidation. While glycolysis is stimulated by glucocorticoids, insulin and AMPK, gluconeogenesis is stimulated by glucocorticoids, but inhibited by insulin and AMPK. Therefore, increased glucocorticoid and insulin levels together with increased lipid influx may ultimately lead to increased AMPK activity in hepatic tissues.

Peripheral lipogenesis occurs in the liver independent of food intake, and endocannabinoids act via the hepatic CB1 receptors to induce hepatic steatosis (Tam et al. 2011). A reversal of this effect has been observed with CB1 receptor antagonism (Gary-Bobo et al. 2007). The activation of the CB1 receptor in mice increases the gene expression of Srebp-1c and results in increased de novo fatty acid synthesis in the liver (Osei-Hyiaman et al. 2005). Cannabinoids may stimulate hepatic lipogenesis through the inhibition of AMPK (Kola et al. 2005). Interestingly, increased baseline hepatic AMPK activity in CB1-KO mice when compared with their WT littermates may be a reflection of the loss of endocannabinoid/CB1-driven hepatic lipogenesis.

CB1-KO mice failed to show an increase in hepatic AMPK activity after glucocorticoid treatment. This might suggest that the CB1 receptor is involved in the AMPK-stimulatory effect of glucocorticoids in the liver.
Alternatively, the reduced adipose tissue stores present in CB1-KO mice might produce less fatty acid influx and stimulation of the liver compared with their WT littermates. It is also possible that the effects of glucocorticoids on the liver are partly mediated through the hypothalamus–vagus circuit, as it is known that central endocannabinoids regulate glucose production in the liver (O’Hare et al. 2011).

There is evidence that glucocorticoids can interact directly with myocardial cells to alter their structure and function (Hadoke et al. 2009). Acute glucocorticoid treatment results in the stimulation of cardiac AMPK (Qi et al. 2006, Puthanveetil et al. 2008). However, with chronic glucocorticoid treatment, we have found reduced cardiac AMPK activity in both rats (Christ-Crain et al. 2008) and mice. AMPK is inhibited by glycogen (McBride et al. 2009); therefore, reduced cardiac AMPK activity may occur in glucocorticoid-treated animals as a result of the increased cardiac glycogen content. AMPK has cardioprotective effects, and reduction of its activity may directly explain the cardiac abnormalities observed in chronic glucocorticoid excess. Long-term inhibition of AMPK has detrimental cardiac consequences. It has been shown that AMPK mediates ischaemic glucose uptake and glycolysis, protects cardiac ATP levels, and limits post-ischaemic cardiac dysfunction (Russell et al. 2004).

Cannabinoids stimulate cardiac AMPK activity (Kola et al. 2005), and this may mediate their cardioprotective effects (Hiley & Ford 2004). In this study, we showed that CB1-KO mice display significantly reduced baseline cardiac AMPK activity when compared with their WT equivalents. This confirms the stimulatory effect of the cannabinoid–CB1 system on cardiac AMPK activity. Similar to glucocorticoid treatment, loss of the CB1 receptor leads to a reduction of cardiac AMPK activity and potentially a detrimental effect on the heart.

It is possible that glucocorticoids have a dual effect on cardiac AMPK activity; there may be a CB1-dependent inhibitory effect and a CB1-independent stimulatory effect. Endocannabinoids have been shown to stimulate cardiac AMPK activity (Kola et al. 2005); therefore, the CB1-dependent inhibitory effect of glucocorticoids on cardiac AMPK activity may result from the inhibition of cardiac endocannabinoid synthesis. One possible explanation for the CB1-independent stimulatory effect of glucocorticoids on cardiac AMPK is the inhibitory effect of glucocorticoids on noradrenaline reuptake in the heart (Grundemann et al. 1998). If noradrenaline reuptake is inhibited, the increased level of noradrenaline can stimulate AMPK activity (Xu et al. 2007). In the WT animals, the CB1-dependent effect is stronger, and this could explain why we observed an inhibition of AMPK activity, while in the CB1-KO mice, we observed only the stimulatory effect.

It is currently unclear how glucocorticoids lead to the activation of the cannabinoid pathway. Di et al. (2003, 2005) have elegantly demonstrated this effect using in vitro studies and have reported a possible membrane receptor-mediated mechanism. More recent studies have confirmed these findings (Wang et al. 2012). Clearly, this aspect requires further studies to elucidate the exact mechanisms involved in glucocorticoid-induced endocannabinoid activation.

In conclusion, our data suggest that an intact cannabinoid pathway is required for the hypothalamic AMPK effects of glucocorticoids, while the adipose tissue changes may occur independent of the CB1 receptor. In addition, knockout of the Cb1 receptor appears to alter the responsiveness of the liver and myocardial tissues to glucocorticoids. These data indicate the importance of the CB1 receptor in the effects of glucocorticoids on various tissues and interaction between glucocorticoids and the novel peripheral CB1 antagonist molecules should be explored.

### 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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