UCP2 regulates the glucagon response to fasting and starvation

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Abstract

Glucagon is important for maintaining euglycemia during fasting/starvation, and abnormal glucagon secretion is associated with type 1 and type 2 diabetes; however, the mechanisms of hypoglycemia-induced glucagon secretion are poorly understood. We previously demonstrated that global deletion of mitochondrial uncoupling protein 2 (UCP2(-/-)) in mice impaired glucagon secretion from isolated islets. Therefore, UCP2 may contribute to the regulation of hypoglycemia-induced glucagon secretion, which is supported by our current finding that UCP2 expression is increased in nutrient-deprived murine and human islets. Further to this, we created α-cell-specific UCP2 knockout (UCP2AKO) mice, which we used to demonstrate that blood glucose recovery in response to hypoglycemia is impaired owing to attenuated glucagon secretion. UCP2-deleted α-cells have higher levels of intracellular reactive oxygen species (ROS) due to enhanced mitochondrial coupling, which translated into defective stimulus/secretion coupling. The effects of UCP2 deletion were mimicked by the UCP2 inhibitor genipin on both murine and human islets and also by [...]
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Elevated basal glucagon levels and reduced hypoglycemia-induced glucagon secretion are underappreciated and poorly understood aspects of type 1 and type 2 diabetes (1–3). Although high plasma glucose normally inhibits glucagon secretion, it remains unclear whether this in vivo response is mediated by glucose sensing, neuronal modulation, paracrine/endocrine control, or a combination thereof (4–10). Uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, is expressed in pancreatic α-cells (11), and its expression can be induced in adipose tissue by a ketogenic diet (12), suggesting a role in the fasting response. While the precise physiological function of UCP2 in islet cells is still debated, it can mildly dissipate the proton motive force generated during mitochondrial electron transport and limit ATP synthesis under certain conditions (13–15). Additionally, UCP2 can limit mitochondrial reactive oxygen species (ROS) production, which can alter associated signaling pathways and/or protect against oxidative stress (16–18). In β-cells, UCP2 deletion elicits only small changes in mitochondrial membrane potential (ΔΨm) with limited effect on ATP (18,19) but rather increases ROS production, which amplifies insulin secretion (18,20). α-Cells, like β-cells, have glucose-sensing machinery that center on KATP channel activity, cellular depolarization, and calcium influx, triggering exocytosis; however, unlike β-cells, they are electrically active and secretory at low glucose concentrations (5,21–24). UCP2 in α-cells could therefore be an important regulator of glucagon secretion via regulation of ATP production, plasma membrane potential, and ROS levels.

Previously, we showed that islets from mice globally lacking UCP2 (UCP2−/−) displayed higher basal glucagon secretion and impaired low glucose–mediated glucagon secretion (11). Due to UCP2's wide expression profile in glucose-sensitive tissues, these changes in α-cell function in UCP2−/− mice could be the result of β-cell and/or extrapancreatic deletion. To decipher the role of UCP2 in α-cells and in the response to fasting, we created an α-cell–specific UCP2 knockout (UCP2AKO) deletion mouse model. These mice displayed reduced fasting plasma glucagon levels and impaired glucagon secretion, due in part to elevated ROS, enhanced glucose-induced hyperpolarization of the ΔΨm, and depolarization of plasma membrane potential. Therefore, we conclude that α-cell UCP2 plays a key role in the hypoglycemic response.
Rhodamine 123, H2O2, and RedTaq polymerase were from Sigma-Aldrich. Genipin was from Wako.

**Immunofluorescent staining.** Dispersed islet cells plated onto glass coverslips were fixed, stained with antibodies, and fluorescence visualized using a laser-scanning confocal microscope (Zeiss 510 LSM) (11).

**Plasma hormone concentration and tolerance tests**

**Insulin tolerance tests.** After a 4-h fast, insulin (1.75 IU/kg body wt) was injected intraperitoneally and blood glucose levels were measured at 0, 15, 30, 60, and 120 min. Plasma glucagon was measured at 0 and 30 min by RIA (Millipore).

**Oral glucose tolerance test.** After a 4-h fast, glucose (3 g/kg body wt) was gavaged and blood glucose levels were measured at 0, 5, 10, 15, 30, 60, and 120 min. Plasma active glucagon-like peptide (GLP)-1 (Meso Scale Discovery, Gaithersburg, MD) was measured at 0 and 10 min.

**Fasting/re-feeding hepatic gluconeogenic gene expression, hepatic glycogen, and plasma metabolites.** Plasma glucose, glucagon, insulin, and corticosterone (MP Biomedicals, Irvine, CA) were measured in fed, 24-h fasted, and 12-h re-fed mice. Mice were killed under fasting or re-fed conditions, and average membrane potentials were calculated across 40 s of steady-state recordings.

**ROS accumulation in isolated islets and its effect on glucagon secretion.** The levels of superoxide and H2O2 were determined using MitosoxRed and 2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), respectively (Molecular Probes; Invitrogen) (35). Fresh isolated islets were preincubated with or without H2O2 (16 μmol/L) or genipin (50 μmol/L) in 1 mmol/L glucose RPMI for 1 h, followed by 1 mmol/L glucose KRB with or without treatments for 1 h before measurement of superoxide or H2O2 levels. Alternatively, islets were cultured overnight in 11 mmol/L glucose RPMI and then H2O2 levels measured with CM-H2DCFDA.

**Quantitative real-time PCR.** Quantitative PCR was performed as previously described (18,32). Primers are listed in Supplementary Table 1.

**Statistics.** Statistical significance was assessed using either the Student t test or a two-way ANOVA for repeated measures followed by a Bonferroni posttest comparison using GraphPad Prism 4. P < 0.05 was considered significant. All data are means ± SEM.

**RESULTS**

**Deletion of α-cell UCP2 impairs glucagon secretion in response to hypoglycemia.** Truncated UCP2, indicative of exon 3 and 4 deletion, was observed only in islets and not in the intestine or hypothalamus of UCP2AKO mice.
(Fig. 1B). Cre (measured by eYFP staining) was expressed in 72 ± 10% glucagon-positive cells (Supplementary Fig. 1B) suggesting efficient deletion. Immunofluorescent staining of dispersed UCP2AKO islets showed cre protein only in α-cells (Fig. 1C), and specific deletion of UCP2 from UCP2AKO α-cells was confirmed by the absence of UCP2 only in glucagon-positive cells (Fig. 1C). Cre (YFP staining) was not expressed in the nucleus of the solitary tract or the distal ileum, reinforcing the specificity of the glucagon promoter for α-cells (Supplementary Fig. 3).

UCP2AKO and Gcg-cre mice displayed similar insulin sensitivities, as the rate and magnitude of blood glucose reduction during an insulin tolerance test were equal (Fig. 2A); however, the rate of blood glucose recovery was slower in UCP2AKO mice, resulting in significantly lower blood glucose at 120 min (Fig. 2A). This correlated with lower glucagon levels 30 min after insulin injection (just before blood glucose levels increased) (Fig. 2B), suggesting that UCP2AKO mice have impaired hypoglycemia-induced glucagon secretion.

Mice lacking glucagon receptors are more glucose tolerant compared with wild-type mice (36). Therefore, we tested whether UCP2AKO mice that display impaired glucagon secretion were more glucose tolerant. An oral glucose tolerance test showed that UCP2AKO mice had similar glucose tolerance to Gcg-cre mice (Fig. 3A) and secreted the same amount of active GLP-1 into plasma (Fig. 3B), suggesting that UCP2AKO mice may preferentially use MCFAs as an energy source under these conditions.

UCP2 expression is increased after nutrient depletion in islets, and α-cell UCP2 deficiency/inhibition impairs glucagon secretion from isolated islets. Human and mouse islets deprived of nutrients (low serum and low glucose) showed similar induction of glucagon and UCP2 expression, highlighting the potential importance of UCP2 during fasting (Fig. 4A and B). We therefore assessed the ability of fresh-isolated UCP2AKO mouse islets to secrete glucagon under low glucose conditions. When conditions were switched from high to low glucose, glucagon secretion was significantly stimulated in Gcg-cre and UCP2AKO islets (P < 0.001) (Fig. 5D); however, the amount secreted by UCP2AKO islets was 52 and 31% lower under high and low glucose concentrations, respectively. Total glucagon content and islet morphology were similar in the two genotypes, suggesting that the reduced secretion from UCP2AKO islets was not due to reduced glucagon content or α-cell number (data not shown).

Gcg-cre islets exposed to genipin, a compound known to inhibit UCP2 (37), secreted significantly less glucagon in the presence of low glucose, and this level was similar to healthy islets.
to UCP2AKO islets in the presence of low glucose alone (Fig. 5E). Conversely, preincubation of UCP2AKO islets with genipin did not alter glucagon secretion (Fig. 5E). For assessment of the interspecies effect of UCP2 inhibition on glucagon secretion, human islets were also exposed to genipin. They secreted significantly less glucagon under low glucose conditions, which was similar to high glucose levels alone (Fig. 5C). Therefore, acute inhibition of UCP2 with genipin reduces glucagon secretion under stimulatory low glucose conditions.

**α-Cell UCP2 deficiency increases glucose-induced ΔΨₘ hyperpolarization, which leads to altered oxidative status and impaired glucagon secretion.** To determine the contribution of UCP2 to mitochondrial coupling in α-cells, we measured changes in glucose-induced ΔΨₘ hyperpolarization (Fig. 6A). Basal (1 mmol/L glucose) ΔΨₘ was similar, but UCP2AKO-YFP α-cells displayed significantly greater glucose-induced hyperpolarization of ΔΨₘ (Fig. 6B).

Even small increases in ΔΨₘ can translate into significantly large increases in mitochondrial ROS production, and recently ROS signaling has emerged as an important regulator of insulin secretion (18,20,38). In contrast, chronic oxidative stress can impair secretion (17,35). As such, we compared the levels of intracellular ROS (H₂O₂ and mitochondrial superoxide) in fresh-isolated islets (to correlate with glucagon secretion studies [Fig. 5D]) and H₂O₂ in cultured islets. No difference in the amount of H₂O₂ but significantly more mitochondrial superoxide was observed in fresh-isolated UCP2AKO islets (Fig. 6C and D). Application of genipin increased H₂O₂ levels 1.37-fold in Gcg-cre islets only (P < 0.01) and increased both H₂O₂ and superoxide levels in human islets (Fig. 6H and I). Therefore, UCP2 deficiency in mouse α-cells results in superoxide buildup and an inability to convert superoxide to H₂O₂.

However, after overnight culture, UCP2AKO islets displayed ~1.7-fold greater intracellular H₂O₂ (Fig. 6E). Cultured Gcg-cre islets appeared to recover from the stress of isolation, as H₂O₂ levels were lower than in fresh-isolated islets (Fig. 6C), suggesting that ROS production is increased in UCP2AKO islets and they experience a chronically altered oxidative status. Whole-islet data were confirmed in dispersed, cultured α-cells, as overall ROS levels were higher in UCP2AKO α-cells (Supplementary Fig. 5).

In addition, treatment of Gcg-cre α-cells with genipin increased ROS to a similar level as UCP2AKO α-cells, whereas genipin had no further effect on UCP2AKO α-cells (Supplementary Fig. 5).

Intracellular ROS levels were correlated with expression of several antioxidant-related genes in fresh isolated whole islets. The expression of superoxide dismutase 2 (Sod2) was ~88% lower in UCP2AKO islets (Fig. 6F), consistent with their inability to convert superoxide to H₂O₂, whereas the H₂O₂-scavenging glutathione peroxidase (Gpx) and catalase (Cat) and the cytoprotective gene, Heme Oxygenase 1 (Ho-1), were unchanged (Supplementary Fig. 6A).

To determine whether increased ROS contribute to impaired glucagon secretion from UCP2AKO islets, we increased intracellular ROS with exogenous H₂O₂ application (Fig. 6C and D). Ggc-cre islets preincubated with H₂O₂ secreted significantly more glucagon under high-glucose and less under low glucose conditions (Fig. 6G). The increased glucagon secretion in the presence of high glucose plus H₂O₂ occurred despite significantly greater insulin secretion (Supplementary Fig. 6C). The addition of H₂O₂ to UCP2AKO islets did not alter glucagon secretion (Fig. 6G). Similarly, the addition of H₂O₂ had no effect on glucose-mediated insulin secretion from UCP2AKO islets (Supplementary Fig. 6C).

Therefore, elevated ROS levels in control islets impaired glucagon secretion similarly to UCP2AKO islets, which experience endogenously higher ROS levels and altered oxidative status.

**Impaired glucagon secretion from UCP2-deficient α-cells correlates with enhanced depolarization of plasma membrane potential and reduced calcium.** Metabolism of glucose and further closure of KₐTT channels reduce glucagon secretion (24). Therefore, we measured KₐTT current and plasma membrane potential. KₐTT currents in Ggc-cre-YFP and UCP2AKO-YFP α-cells could be similarly activated and inhibited by diazoxide and tolbutamide, respectively (Supplementary Fig. 7A). However, despite a similar magnitude of change in plasma membrane potential when switched from high to low glucose, the plasma membrane was more depolarized in UCP2AKO-YFP α-cells under both conditions (Fig. 7A and B).

Preincubation of Ggc-cre-YFP α-cells with high glucose plus genipin increased (depolarized) plasma membrane potential...
but had no further effect in UCP2AKO-YFP α-cells (Fig. 7C). Therefore, the absence of UCP2 in α-cells shifts the operating range of plasma membrane potentials, which might explain the impaired glucagon secretion.

The more depolarized plasma membrane potential in the presence of high glucose suggests that α-cell UCP2 deficiency may increase ATP levels and close/inactivate K_{ATP} channels. There was a trend toward increased ATP content in UCP2AKO islets under high glucose conditions (Supplementary Fig. 7B); however, any effect of α-cell UCP2 deletion may be masked by β-cell ATP content. Therefore, we determined whether the K_{ATP} channel
opener, diazoxide, could overcome the impaired secretion. Under high glucose conditions, the addition of low-dose diazoxide (1 μmol/L) increased glucagon secretion from UCP2AKO islets to a level similar to that in Gcg-cre islets without diazoxide; however, the UCP2AKO islets were unable to reach a level of secretion as high as that in Gcg-cre islets in the presence of 1 μmol/L diazoxide (Fig. 7D). Conversely, under low glucose conditions, low-dose diazoxide increased glucagon secretion from UCP2AKO islets to a level similar to that in Gcg-cre islets in the absence of diazoxide. The addition of more diazoxide dose-dependently decreased secretion (Fig. 7D), which was similar to previous observations (24). Therefore, under low glucose conditions opening K<sub>ATP</sub> channels can correct the glucagon secretion defect observed in UCP2AKO islets.

Influx of calcium is required for low glucose–mediated exocytosis of glucagon (39), and thus we examined changes in cytosolic calcium. The amount of intracellular calcium under high and low glucose conditions was reduced in YFP-UCP2AKO<sub>a</sub>-cells (Fig. 7F). Pretreatment of YFP-Gcg-cre cells with genipin also significantly reduced intracellular calcium levels, whereas H<sub>2</sub>O<sub>2</sub> had no effect (Fig. 7F). In addition, intracellular calcium in UCP2AKO α-cells oscillated faster under low glucose conditions (Supplementary Fig. 8E). Reduced cytosolic calcium in UCP2AKO α-cells reflects an impaired ability to secrete glucagon.
FIG. 6. UCP2AKO α-cells display enhanced hyperpolarization of ΔΨm and increased superoxide levels. A: Representative traces of ΔΨm (Mmp) relative fluorescent unit (RFU) measurements in YFP+ α-cells. B: ΔΨm (Mmp) was measured in high glucose (20 mmol/L) (Δ1) and NaN3 (5 mmol/L) (Δ2) and normalized to basal fluorescence in the presence of low glucose (1 mmol/L). N = 28–29 cells from 6 mice/genotype. *P < 0.05. C and D: Fresh-isolated islets were cultured with or without H2O2 (16 μmol/L) or genipin (50 μmol/L) before measurement of H2O2 (CM-H2-DCFDA) (C) or superoxide (MitosoxRed) (D). N = 20–28 islets, 6–8 mice/genotype. *P < 0.05, **P < 0.01. E: Islets were cultured overnight, and then intracellular H2O2 (CM-H2-DCFDA) was measured. N = 19–27 islets, 5–7 mice/genotype. *P < 0.05. F: Islet Sod2 expression. N = 4–5 mice/genotype. *P < 0.05. G: Glucose-inhibited glucagon secretion with or without H2O2 (16 μmol/L). Secretion is per microgram of DNA, normalized to Gcg-cre high glucose (HG) conditions. LG, low glucose. N = 3–11 mice/genotype. *P < 0.05, **P < 0.01. H and I: Human islets were incubated with 50 μmol/L genipin for 2 h before measurement of H2O2 (CM-H2-DCFDA) (H) and superoxide (MitosoxRed) (I) levels. N = 4 individual donors. *P < 0.05, **P < 0.01. 
DISCUSSION

α-Cells secrete glucagon in response to low blood glucose and are inhibited when blood glucose levels rise (24). We now show that UCP2 expression is increased in islets deprived of nutrients, α-cell UCP2 is necessary for appropriate glucagon secretion, and the absence of UCP2 impairs α-cell function. UCP2AKO mice displayed impaired glucagon secretion in vivo, which inhibited the hepatic response to hypoglycemia (40), including reduced expression of the hepatic gluconeogenic genes, Pepck and G6pc, and higher glycogen levels. Reduced fasting levels of plasma MCFAs in UCP2AKO mice suggest compensation for impaired hepatic gluconeogenesis and increased usage of these fatty acids during hypoglycemia. MCFAs are absorbed directly through the portal vein, making them a direct energy source for the liver (41). MCFAs also produce more ketones per unit of energy than long-chain fatty acids, which could provide crucial energy for the brain during nutrient depletion and when gluconeogenesis is impaired, such as in fasted UCP2AKO mice. Similarly, mice globally lacking glucagon receptors demonstrate impaired activation of hepatic gluconeogenic genes and

FIG. 7. UCP2AKO α-cells have more depolarized plasma membranes and reduced intracellular calcium. A: Representative traces of plasma membrane potential in YFP+ α-cells incubated in high (20 mmol/L) and low (1 mmol/L) glucose. B: Average membrane potential in YFP+ α-cells. N = 5–8 cells, 3 mice/genotype. *P < 0.05. C: Average plasma membrane potential measured in YFP+ α-cells (N = 8–13) with or without genipin (50 μmol/L), and at least 3 mice/genotype. *P < 0.05, **P < 0.01. D: Glucose-inhibited glucagon secretion from islets incubated with or without diazoxide (1, 10, and 100 μmol/L) in the presence of high glucose (20 mmol/L) (i) or low glucose (1 mmol/L) (ii). *P < 0.05. N = 3 mice/genotype. E: Incremental area under the curve (IAUC) for cytosolic calcium, normalized to high glucose plus arginine levels. Representative traces are shown in Supplementary Fig. 8. *P < 0.05. N = 19–40 cells/treatment, 3 mice/genotype.
enhanced lipogenesis, yet have improved glucose tolerance (36,42). Interestingly, UCP2AKO mice, similar to adult mice with 98% α-cell ablation (43), displayed normal glucose tolerance. Therefore, we show that appropriate glucagon secretion is required for normal hepatic function during fasting and that α-cell UCP2 deficiency disrupts this process, solidifying the role of UCP2 in the α-cell response to hypoglycemia.

The amount of glucagon secreted from UCP2AKO islets under stimulatory conditions was the same as that secreted from Gcg-cre islets under inhibitory conditions. Preincubation of Gcg-cre islets with the UCP2 inhibitor genipin reduced low glucose-mediated glucagon secretion by a magnitude similar to that in UCP2 deletion, whereas genipin did not further reduce glucagon secretion from UCP2AKO islets, suggesting its relative specificity of action for UCP2. Interestingly, the glucagon-lowering effect of genipin was not isolated to mouse islets, as it also significantly impaired low glucose-mediated glucagon secretion from human islets. Insulin released from β-cells lowers glucagon secretion via paracrine inhibition (5,7,9); however, the reduced glucagon secretion from UCP2AKO islets was not explained by this mechanism, as insulin secretion was similar from Gcg-cre and UCP2AKO cultured islets (Supplementary Fig. 6B). Therefore, the presence of UCP2 in α-cells is directly required for appropriate glucagon release.

The α-cell secretes glucagon within a narrow range of plasma membrane potentials (24) and is sensitive to small changes in ATP concentration (22), and it is suggested that glucose metabolism does not accelerate ATP synthesis in this cell type (44). We previously showed that UCP2 is expressed at higher levels in α compared with clonal β-cell lines (11). Thus, high expression of UCP2 may normally limit ATP production in α-cells via an uncoupling role. Furthermore, small interfering RNA knockdown of UCP2 in clonal α-cells increased ATP levels (11). Although no difference in ATP concentrations in UCP2AKO versus Gcg-cre islets was detected, the increased glucose-induced hyperpolarization of ΔΨm and more depolarized plasma membrane of UCP2AKO α-cells suggest increased ATP synthesis. Recent studies show that depolarization of α-cells inhibits the activity of Na+ and Ca2+ channels resulting in cessation of glucagon secretion (22,24), and we show reduced intracellular calcium levels in UCP2AKO α-cells under both high and low glucose conditions. Similarly, exposure of Gcg-cre α-cells to genipin depolarized the plasma membrane and reduced calcium entry. As such, we propose that UCP2AKO α-cells have more closed KATP channels, limiting their glucagon secretion capacity (24,45). It was no surprise that the addition of low-dose diazoxide, which opens the KATP channel, corrected this impairment under low glucose conditions. Conversely, in the presence of high glucose, low-dose diazoxide increased glucagon secretion from UCP2AKO islets to a level similar to basal secretion from Gcg-cre islets, yet further addition of diazoxide did not elevate secretion from UCP2AKO islets. These data suggest that altered membrane potential is not the only factor limiting glucagon secretion from UCP2AKO islets under high glucose conditions.

It has been proposed that generation of low-level ROS, likely H2O2, amplifies insulin secretion (20,46), and recently,
we showed that β-cell–specific deletion of UCP2 increases insulin secretion in an ROS-dependent manner (18). We now show that the acute addition of exogenous H₂O₂ to control islets increased glucagon secretion under high glucose and reduced glucagon secretion under low glucose conditions, a situation reminiscent of the disordered glucagon secretion from murine UCP2−/− islets (11) and patients with diabetes (1) and human islets exposed to genipin. It is therefore conceivable that UCP2 regulates the levels of ROS in α-cells and, in turn, glucagon secretion. Acute inhibition of UCP2 in Gcg-cre islets with genipin increased endogenous H₂O₂ levels, which correlated with reduced low glucose–mediated glucagon secretion. Similarly, UCP2AKO islets had elevated mitochondrial superoxide levels and signs of chronically altered redox-related signaling pathways (i.e., ROS levels remained high after overnight culture and altered antioxidant gene expression [including reduced Sod2 expression] was observed), which also correlated with impaired glucagon secretion. Therefore, elevated intracellular ROS can reduce low glucose–mediated glucagon secretion, although the specific ROS species and its signaling targets are unclear.

We propose that α-cell UCP2 expression is normally increased in response to low nutrient levels and limits ∆Ψm hyperpolarization, which, by limiting ATP and/or ROS production, contributes to the cells’ ability to secrete glucagon. Therefore, α-cell–specific deletion of UCP2 results in greater depolarization of the plasma membrane and, although still responsive to changes in glucose, reduces calcium entry and lowers overall secretion capacity (Fig. 8A). Deletion of α-cell UCP2 disrupted the hypoglycemic response in mice, implying that increased UCP2 expression/activity, such as that which occurs after fasting, is required to promote glucagon secretion and is protective in this role (Fig. 8B). Conversely, elevated expression or activity of UCP2 may also contribute to the dysregulated glucagon secretion that is observed in the diabetic state.

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E.M.A. researched data, contributed to discussion, wrote the manuscript, and reviewed and edited the manuscript. C.A.R.-D., K.J.P., and A.B.H. researched data, contributed to discussion, and reviewed and edited the manuscript. S.S. researched data and contributed to discussion. H.Y.G. contributed to discussion and reviewed and edited the manuscript. D.K. researched data and contributed to discussion. P.G., P.L.H., and B.B.L. contributed to discussion and reviewed and edited the manuscript. M.B.W. designed the study, contributed to discussion, and reviewed and edited the manuscript. M.B.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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