Considerable debate exists about whether alterations in mitochondrial respiratory capacity and/or content play a causal role in the development of insulin resistance during obesity. The current study was undertaken to determine whether such alterations are present during the initial stages of insulin resistance in humans. Young (~23 years) insulin-sensitive lean and insulin-resistant obese men and women were studied. Insulin resistance was confirmed through an intravenous glucose tolerance test. Measures of mitochondrial respiratory capacity and content as well as H$_2$O$_2$ emitting potential and the cellular redox environment were performed in permeabilized myofibers and primary myotubes prepared from vastus lateralis muscle biopsy specimens. No differences in mitochondrial respiratory function or content were observed between lean and obese subjects, despite elevations in H$_2$O$_2$ emission rates and reductions in cellular glutathione. These findings were apparent in permeabilized myofibers as well as in primary myotubes. The results suggest that reductions in mitochondrial respiratory capacity and content are not required for the initial manifestation of peripheral insulin resistance.

Despite an alarming increase in the prevalence of diet-induced insulin resistance or prediabetes, the underlying etiology at the biochemical level remains unclear and heavily debated. With respect to skeletal muscle, reductions in ATP synthase activity and capacity (1–6) and/or mitochondrial content (2,7) have been suggested as potential causes of insulin resistance. Although several reports have demonstrated lower ATP synthase flux and synthesis capacity (1–6) and/or mitochondrial content (1,2,7–11) in the presence of insulin resistance and/or overt type 2 diabetes, considerable debate remains about whether such differences are causal, consequential, or unrelated to insulin resistance. Establishing causation within a given pathophysiological process requires adherence to certain general criteria: 1) Associative data must consistently relate a particular stimulus with a disease, 2) the stimulus must precede disease onset, and 3) removal of the stimulus must prevent or reverse the disease.
With respect to the first criterion, lower ATP synthase flux assessed in vivo with $^{31}$P magnetic resonance spectroscopy as well as a lower ATP generating capacity determined ex vivo in isolated mitochondria and/or permeabilized fibers in the presence of obesity-related insulin resistance have been reported by some (1–6), but not all (9–15), investigators. Discrepancies likely reflect differences in age (16) (range ~23–60 years) and severity of fasting hyperglycemia/insulinemia between subject pools (17) as well as in the methodologies used to quantify oxidative phosphorylation (18,19). A similar degree of heterogeneity exists in relation to mitochondrial content, with close to an equal number of investigations reporting lower (1,2,7–11) or no difference (3,12–14,20) in insulin-sensitive lean versus insulin-resistant obese subjects. The few studies that attempted to correlate indices of oxidative phosphorylation and/or mitochondrial content with insulin sensitivity did not find a significant relationship (12–14), but one did (8). Regarding the second criteria, acutely elevating plasma free fatty acids through lipid infusion (3–6 h) has been shown to transiently depress skeletal muscle insulin sensitivity in humans without affecting mitochondrial function and/or content (21,22). Moreover, results from rodent models of high-fat diet–induced insulin resistance have consistently demonstrated an initial upregulation in mitochondrial capacity for oxidative phosphorylation as well as content despite the presence of insulin resistance (23–25). Finally, regarding the third criterion, administration of an iron-deficient diet in rodents failed to induce insulin resistance despite stark reductions in electron transport system (ETS) protein content (26). Taken together, these data from humans coupled with a large body of evidence in rodent models do not support altered capacity for oxidative phosphorylation and/or mitochondrial content as an underlying cause of diet-induced insulin resistance (23–26).

Mitochondria contribute to the regulation of a number of cellular functions beyond providing energy, including cellular redox balance. Elevated mitochondrial oxidant emission stemming from nutrient overload has been put forth as a potential primary event in the etiology of diet-induced insulin resistance (27–29) based in part on observations of higher mitochondrial H$_2$O$_2$ emitting potential and oxidation of the cellular glutathione pool in skeletal muscle of insulin-resistant obese compared with insulin-sensitive lean subjects (9,27). The current study was undertaken to determine whether differences in mitochondrial respiratory capacity and content as well as in the cellular redox environment are detectable during the early stages of impaired glucose tolerance in humans. Similar levels of mitochondrial respiratory capacity and content were found in permeabilized myofibers and cultured primary myotubes from young (~23 years) insulin-sensitive lean and insulin-resistant obese male and female subjects, despite higher mitochondrial H$_2$O$_2$ emitting potential and lower whole-cell glutathione content in obese subjects. Taken together, these data suggest that defects in mitochondrial capacity for oxidative phosphorylation (either inherent or acquired) are not required for the development of obesity-induced insulin resistance in humans but are consistent with the proposed redox-regulated control of insulin sensitivity.

**RESEARCH DESIGN AND METHODS**

**Human Subjects, Tissue Biopsy, Intravenous Glucose Tolerance Test, and Primary Human Cell Culture**

Lean (n = 20, 10 male, 10 female) and obese (n = 20, 10 male, 10 female) subjects 18–35 years of age were recruited from the faculty and student population of the East Carolina University. All subjects were sedentary as defined by self-report responses to the International Physical Activity Questionnaire (30). Inclusion criteria were BMI $<25$ kg/m$^2$ (lean subjects) and $\geq 30$ kg/m$^2$ (obese subjects). Exclusion criteria were elevated fasting serum glucose (>100 mg/dL) or total cholesterol (>200 mg/dL) levels and the presence of metabolic disease, diabetes, heart disease, or pregnancy. The university’s Institutional Review Board for human subjects approved all procedures used in this study, and all subjects signed a written consent. All female subjects were studied within the first 5 days of the follicular phase of their menstrual cycle to avoid the potential confounding influence of progesterone on mitochondrial function (31). On the day of the experiment, subjects reported to the clinical facility after an overnight fast (~10 h). After resting for 20 min, a catheter was placed in the antecubital vein, and a baseline blood sample was obtained. A skeletal muscle biopsy specimen was then obtained from the vastus lateralis by the needle biopsy technique as described previously (27,32) followed by an intravenous glucose tolerance test (IVGTT) to determine insulin sensitivity (33). Percent body fat was determined by dual-energy X-ray absorptiometry (GE Lunar Prodigy Advanced). Blood insulin level was assessed by electrochemiluminescence immunoassay (LabCorp).

A second cohort of subjects comprising 10 young (21.1 ± 1 years) lean (BMI $<25$ kg/m$^2$) males and 10 young (25.6 ± 3 years) obese (BMI $\geq 30$ kg/m$^2$) males were recruited for primary human skeletal muscle cell culture. After a 10-h overnight fast, ~50–100 mg of skeletal muscle from the vastus lateralis was obtained by percutaneous biopsy. The isolation and culturing of human primary skeletal muscle cells from biopsy specimens was performed as previously described (34). On day 7 of differentiation, cells were incubated for 24 h in either differentiation media (Dulbecco’s modified Eagle’s medium 5 mmol/L glucose [control]) or differentiation media supplemented with 10 mmol/L galactose and then harvested for respirometry experiments. Separate
aliquots of the same passage number were grown and treated similarly and then harvested for analysis of glutathione, mitochondrial proteins, and citrate synthase activity.

**Preparation of Permeabilized Muscle Fibers and Primary Myotubes**

A portion of each muscle sample was separated for preparation of permeabilized fiber bundles as described previously (32), with the remainder quick frozen and stored in liquid nitrogen. Fiber bundles (0.2–0.8 mg dry wt) were separated along their longitudinal axis with a pair of needle-tipped forceps under magnification (MX6 Stereoscope; Leica Microsystems, Buffalo Grove, IL). Bundles were then treated with saponin 30 μg/mL for 30 min at 4°C and subsequently washed in cold buffer Z containing 105 mmol/L K-MES [potassium salt of 2-(N-morpholino)ethanesulfonic acid], 30 mmol/L KCl, 1 mmol/L EGTA, 10 mmol/L K₂HPO₄, 5 mmol/L MgCl₂ · 6H₂O, 0.005 mmol/L glutamate, and 0.002 mmol/L malate with 5.0 or 0.5 mg/mL BSA (pH 7.1) until analysis (<1 h).

Myotubes were permeabilized as previously described (35). Cells were washed with PBS and lifted from culture flasks with 0.05% trypsin EDTA. This reaction was neutralized by adding 10% FBS to the cell suspension and centrifuged for 10 min at 1,000 rpm at room temperature. The cell pellet was then resuspended in growth media. Calculated glucose utilization and lactate levels were normalized to cell count.

Mitochondrial Respiration and H₂O₂ Measurements

High-resolution O₂ consumption measurements were conducted at 30°C (fibers) or 37°C (myotubes) with the OROBOROS Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria). Permeabilized fiber bundles were incubated for 5 min in 10 mmol/L pyrophosphate before assay to deplete all endogenous adenine nucleotides and to inhibit contraction of the fibers during the assay. H₂O₂ emission was determined as previously described (27). At the conclusion of each experiment, fibers were washed in double-distilled H₂O to remove salts, freeze-dried, and weighed. The supernatant was used for analysis.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed by t tests or one-way ANOVA with
RESULTS

Subject Characteristics

Young men (22 ± 1 years) and women (23 ± 1 years) were recruited and subsequently grouped according to BMI as either lean (23.8 ± 0.5 kg/m²) or obese (36.7 ± 1.1 kg/m²). Baseline subject characteristics are shown in Table 1. Fasting blood glucose was similar between groups; however, corresponding insulin and homeostasis model assessment for insulin resistance values were significantly elevated in the obese subjects regardless of sex. The insulin sensitivity index calculated from the IVGTT was significantly lower in both obese male and obese female subjects (Fig. 1), confirming insulin resistance in this subject group.

Both Sex and Obesity Do Not Affect Mitochondrial Respiratory Capacity

Mitochondrial oxygen consumption was assessed in permeabilized fiber bundles prepared from skeletal muscle biopsy specimens obtained after an overnight fast. In the presence of saturating concentrations of glutamate and malate, both basal (state 4) and maximal ADP (state 3)-supported respiration were not different on the basis of sex or obesity (Fig. 2A). Because no effect of sex was found for insulin sensitivity (Fig. 1) or respiratory capacity (Fig. 2A), all remaining data from male and female subjects were pooled according to BMI. The combination of glutamate and malate provides electrons exclusively at the level of complex I. To assess electron transfer capacity throughout the entire system, saturating concentrations of substrates directed at β-oxidation (palmitoyl-l-carnitine), complex I (malate, glutamate), and complex II (succinate) were added sequentially in the presence of maximal ADP. All respiration experiments were performed in the presence of 20 mmol/L creatine in an effort to clamp ADP at the desired concentration throughout each experiment. In agreement with what was observed with glutamate and malate, respiration rates were once again not different between lean and obese subjects (Fig. 2B). Both maximal uncoupled respiration and protein content of various components of the oxidative phosphorylation system (OXPHOS) were not different between lean and obese subjects, suggesting that mitochondrial density was similar between groups (Fig. 2C).

Obesity Does Not Affect Respiratory Capacity or Mitochondrial Content Within Primary Human Myotubes

As observed in the permeabilized fibers, respiratory capacity was also not different in permeabilized myotubes from young lean versus obese subjects (Fig. 3A). Succinate-supported respiration (electron entry through complex II exclusively) was determined in the presence of rotenone under basal, maximal ADP, and uncoupled (FCCP) conditions (Fig. 3B). No significant differences were observed between lean and obese myotubes. In agreement with data from frozen muscle samples, neither OXPHOS protein content (Fig. 3C) nor citrate synthase activity (Fig. 3D) differed between lean and obese subjects.

Acute Exposure to Galactose Elevates Respiratory Capacity in Primary Myotubes: No Impact of Obesity

In contrast to skeletal muscle tissue, which in the basal state relies on oxidative phosphorylation to meet the majority of its energetic needs, myocytes in culture rely on glycolysis almost exclusively for ATP production. Replacing glucose with galactose in culture media has previously been shown to increase mitochondrial content, morphology, and oxidative capacity presumably as a consequence of increased reliance on oxidative phosphorylation within the galactose-grown cells (36). To determine potential differences in adaptability between lean and obese cells, myotubes were incubated for 24 h in the presence of galactose. Exposure to galactose elevated mitochondrial respiratory capacity (Fig. 4B, pooled lean and obese data) within myotubes prepared from lean and

Table 1—Basic clinical characteristics of the study groups

|                        | Lean (n = 20) | Obese (n = 20) | Lean (n = 10) | Obese (n = 10) |
|------------------------|--------------|---------------|--------------|---------------|
| Age (years)            | 22 ± 1       | 25 ± 2        | 23 ± 1       | 24 ± 2        |
| BMI (kg/m²)            | 23.8 ± 0.6   | 37.7 ± 1.3*   | 23.1 ± 0.6   | 38.5 ± 1.7*   |
| Body fat (%)           | 21.6 ± 2.2   | 38.9 ± 1.4*   | 34.2 ± 1.4#  | 49.1 ± 1.2*   |
| Fasting plasma glucose (mg/dL) | 84.8 ± 1.4   | 88.2 ± 2.1    | 82.2 ± 1.6   | 89.8 ± 1.9*   |
| Fasting plasma insulin (µU/mL) | 4.5 ± 0.5    | 15.8 ± 1.9*   | 5.7 ± 0.6    | 17.2 ± 4.0*   |
| HOMA-IR (mmol · mU/L⁻¹²) | 1.1 ± 0.1    | 4.0 ± 0.9*    | 1.2 ± 0.1    | 3.9 ± 1.0*    |

Data are mean ± SEM. HOMA-IR, homeostasis model assessment for insulin resistance. *Different from lean (P < 0.05). #Different from lean male.
obese subjects (Fig. 4A). It should be noted that this galactose-induced elevation in respiratory capacity was evident despite the continual presence of glucose in the culture media. To confirm that the addition of galactose resulted in an increased reliance on oxidative metabolism, substrate incubation experiments were repeated while simultaneously tracking lactate appearance and glucose disappearance within and from the culture media. As expected, glucose utilization (Fig. 5A, pooled data from lean and obese subjects) and lactate appearance (Fig. 5B, pooled data from lean and obese subjects) were lower in the presence of galactose compared with control conditions. Assessment of basal and FCCP-induced respiration within intact myotubes also revealed no differences between lean and obese subjects (Fig. 5C). When data for lean and obese myotubes were pooled, elevations in respiration were evident in the presence of galactose compared with control conditions; however, significance was only observed under FCCP-stimulated conditions (Fig. 5D).

**Elevations in Mitochondrial H$_2$O$_2$ Emission and Reductions in Cellular Glutathione During Obesity**

To determine the impact of obesity on mitochondrial redox homeostasis, H$_2$O$_2$ emitting potential was assessed in permeabilized myofibers prepared from human subjects under saturating substrate (palmitoyl-L-carnitine 25 μmol/L, malate 2 mmol/L, glutamate 5 mmol/L, and succinate 10 mmol/L) conditions in the absence of ADP. In agreement with previous findings (27), H$_2$O$_2$ emission rate was higher in fibers prepared from obese subjects (Fig. 6A). Reductions in total glutathione were also evident in the obese subjects in both skeletal muscle homogenate (Fig. 6B) and myotube lysate (Fig. 6C).

**DISCUSSION**

The impetus for the present investigation stems from the ongoing debate within the field about whether detriments in mitochondrial oxidative phosphorylation capacity and/or content are detectable under conditions of obesity-related insulin resistance as well as whether such derangements exist as a potential cause of the condition. Confirmation of this hypothesis would require impairments in mitochondrial phosphorylation capacity and/or content during obesity to be present at or near the onset of metabolic disease (e.g., in young insulin-resistant obese subjects without substantial elevations in fasting blood glucose levels). The current study design was developed on the basis of this concept. The present findings reveal no differences in mitochondrial respiratory capacity or content between young lean and young obese subjects. This is supported by experiments conducted with permeabilized myofibers and primary myotubes as well as with intact myotubes in culture. Although indices of mitochondrial respiratory capacity were unaffected by obesity, higher mitochondrial H$_2$O$_2$ emitting potential was observed in the obese subjects. Moreover, total cellular glutathione was found to be lower in both skeletal muscle homogenate and myotube lysate derived from obese subjects. Taken together, these data provide evidence that derangements in mitochondrial respiratory capacity are not required for insulin resistance, whereas total cellular redox buffering capacity appears to be impaired in humans at the early stages of obesity-related insulin resistance consistent with the latter contributing to the etiology of metabolic disease.

The present findings agree with two other investigations of a similar study design in which rates of mitochondrial oxygen consumption from isolated mitochondria were not found to differ between lean and obese humans (9,12). In contrast, Larsen et al. (10) reported differences in respiratory capacity between lean and obese nondiabetic subjects; however, these differences were no longer evident when rates of respiration were normalized to citrate synthase activity. The findings of Larsen et al. agree with a large body of evidence identifying lower mitochondrial content in obese compared with lean humans (1,2,7–11). In these studies, the age range of the subject populations tested is important to point out. The majority of lean versus obese comparisons have been made in subject populations >30 years of age (1,2,7–11). Reductions in ex vivo mitochondrial ATP production, citrate synthase activity, and mitochondrial protein abundance have been observed as a function of age in otherwise healthy humans (16). In the present investigation, depressions in mitochondrial content (assessed by Western blotting, maximal FCCP-supported respiration, and citrate synthase activity) were not evident within young (~23 years) obese subjects. To our knowledge, only one other study has assessed mitochondrial content in an obese, insulin-resistant, ~22-year-old population (12). In agreement with the current study, no differences in mitochondrial DNA copy number were found between lean and obese subjects (12). It should be emphasized that although both citrate
synthase activity and Western blotting analyses of complex I–V have recently been shown to correlate strongly with transmission electron microscopy as surrogate indices of mitochondrial content (37), such measures do not rule out the possibility for reductions in specific mitochondrial proteins. In line with this notion, a study that incorporated tandem mass spectroscopy demonstrated lower abundance of specific mitochondrial proteins per mitochondrial mass within insulin-resistant obese subjects (9). Proteins included subunits within complex I as well as enzymes involved in the oxidation of branched-chain amino acids and fatty acids. The authors also reported elevations in mitochondrial H$_2$O$_2$ emitting potential within the obese subjects, which was suggested to result from elevated reducing pressure within the ETS as a consequence of reduced ETS protein components relative to normal tricarboxylic acid cycle flux. Such conditions would be expected to favor higher NADH/NAD$^+$ for a given rate of respiration, thereby promoting accelerated electron leak (38,39) and potentially explaining the increase in H$_2$O$_2$ emission observed ex vivo under saturating substrate conditions. Assessment of mitochondrial protein abundance through tandem mass spectroscopy was not performed in the present investigation; thus, is remains to be seen whether similar alterations in specific ETS proteins are apparent in young insulin-resistant obese populations.

An alternative explanation for the elevated H$_2$O$_2$ emitting potential associated with obesity involves peroxide-mediated alterations to redox buffering integrity. Our group has previously reported higher H$_2$O$_2$ emitting potential in the presence of obesity as well as in otherwise healthy humans 4 h after a single high-fat meal (27), thus demonstrating the sensitivity of the ETS to positive metabolic balance. Elevations in substrate supply in the absence of a concomitant increase in demand for ATP generation (i.e., high caloric diet under sedentary conditions) is expected to increase NADH/NAD$^+$, elevate reducing pressure within the ETS, and thus accelerate electron leak (38,39). The glutathione and thioredoxin

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**Figure 2**—Mitochondrial respiratory capacity is not different in permeabilized myofibers from young lean and obese humans. A and B: Mitochondrial oxygen consumption rate (J$_{O2}$) was assessed in permeabilized myofibers prepared from vastus lateralis muscle of lean and obese human subjects. A: J$_{O2}$ in the presence of glutamate (10 mmol/L) and malate (2 mmol/L) (GM) under basal (state 4 [GM$_4$]) and maximal ADP (4 mmol/L)-stimulated (state 3 [GM$_3$]) conditions. B: J$_{O2}$ in response to palmitoyl-L-carnitine (25 μmol/L) and malate (2 mmol/L) (PCM), ADP (4 mmol/L), cytochrome C (Cyto C) (10 μmol/L), glutamate (G) (10 mmol/L), succinate (S) (10 mmol/L), and FCCP (2 μmol/L). With the exception of A, male and female data were pooled to compare lean vs. obese. C: Western blot analysis of mitochondrial OXPHOS proteins (MitoSciences) prepared from vastus lateralis frozen tissue homogenate. Data are mean ± SEM; n = 7–10 (A); n = 16–17 (B); n = 10 (C). CI, complex I; CII, complex II; CIII, complex III; CV, complex V; FL, female lean; FO, female obese; L, lean; ML, male lean; MO, male obese; O, obese; wt, weight.
redox buffering systems operating within the matrix are responsible for degrading the H₂O₂ produced. It is possible that prolonged exposure to increased H₂O₂ may compromise redox buffering integrity similar to that observed in the current study for whole-cell reduced glutathione. It should be emphasized that the lack of a repeated-measures design in the current investigation prevented us from establishing causation; however, because alterations in H₂O₂ emitting potential and glutathione were evident during the early stages of obesity-induced insulin resistance, these data support a potential causative role for altered cellular redox in contributing to disease etiology.

The current study was conducted to determine the relationship between insulin sensitivity and mitochondrial oxidative capacity and content as well as redox homeostasis, specifically within skeletal muscle. Insulin sensitivity was determined by way of the IVGTT, which although it has been shown to correlate strongly with that of the hyperinsulinemic-euglycemic clamp technique in humans (40), the insulin sensitivity index measure is derived from the combined effect of insulin on both skeletal muscle and liver (33). This is a limitation of the current investigation because mitochondrial respiratory capacity and content were not determined in liver mitochondria.

The current results illustrating similar levels of mitochondrial respiratory capacity and content between primary human myotubes prepared from lean and obese subjects agree with previously published reports (41). In the current study, acute exposure of differentiated myotubes to galactose led to similar increases in maximal respiration in both lean and obese myotubes. These results contrast with a recent report (41) in which the response to 24-h lipid exposure was found to be blunted in obese compared with lean myotubes. This discrepancy is most likely a result of differences between permeabilization strategies between the two studies. In the previous study (41), permeabilization was carried out directly in the oxygraph chamber with twofold higher digitonin concentration and without the inclusion of a subsequent wash step. The inclusion of a wash step following digitonin permeabilization is necessary to remove endogenous substrates that may interfere with...
rates recorded in response to exogenous substrate additions (42).

In conclusion, the results of the current study do not support the widely held hypothesis that diet-induced insulin resistance may be caused by alterations in mitochondrial oxidative capacity or content. These findings in conjunction with those of other studies (9,12–14) instead suggest that any such changes in mitochondrial oxidative capacity or content.

Figure 4—Myotubes from young lean and obese humans show similar adaptive increases in respiratory capacity in response to metabolic challenge. A and B: Fully differentiated myotubes were incubated for 24 h in the presence of galactose, which was added directly to the differentiation media. After this 24-h incubation, myotubes were harvested and permeabilized, and oxygen consumption was assessed. B: Data from lean and obese subjects were pooled to illustrate the effects of galactose. Data are mean ± SEM; n = 9–10 (A); n = 19 (B). *Different from vehicle control (P < 0.05). Cyto C, cytochrome C; G, glutamate; JO2, rate of mitochondrial oxygen consumption; M, malate; PC, palmitoyl-L-carnitine; S, succinate.

Figure 5—Basal and FCCP-stimulated respiration within intact primary human myotubes. Primary human myotubes were incubated for 24 h in differentiation media alone (control) or in differentiation media supplemented with galactose. A and B: Glucose utilization and lactate production during the 24-h incubations. C and D: Basal and FCCP (5 μmol/L)–stimulated respiration was assessed in intact primary human myotubes after the 24-h incubation. D: Pooled data from lean and obese subjects. Data are mean ± SEM; n = 14–16 (A, B, and D); n = 7–8 (C). *Different from corresponding vehicle control condition (P < 0.05). AU, arbitrary units; Con, control; Gal, galactose; JO2, mitochondrial oxygen consumption rate.
volume and/or function observed in response to diet-induced obesity are most likely secondary to the initial derangements in peripheral insulin sensitivity. This concept agrees well with data from rodent models in which high-fat feeding has been shown to induce peripheral insulin resistance despite initial adaptive increases in mitochondrial respiratory capacity and content (23,24). In contrast to that observed for indices of mitochondrial function and content, elevations in H₂O₂ emitting potential as well as alterations in the glutathione pool were readily apparent in young insulin-resistant obese subjects. At present, it would seem that therapeutic strategies directed at preventing the onset of insulin resistance would be best served by targeting the restoration of the peripheral metabolic balance (through decreasing nutrient supply or increasing energetic demand) and/or the preservation of mitochondrial redox buffering integrity.

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**Author Contributions.** K.H.F.-W. researched data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. T.M.W. and L.A.A.G. researched data and reviewed and edited the manuscript. B.L.C., P.M.B., C.L.K., and J.M.M. researched data. T.P.G., T.M.W. and L.A.A.G. researched data and reviewed and edited the manuscript. P.D.N. contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. P.D.N. 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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