Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle

Ahmad Mujahid, Yukio Akiba, and Masaaki Toyomizu

Nutritional Biochemistry of Animals, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai, Japan

Submitted 2 December 2008; accepted in final form 17 June 2009

Mujahid A, Akiba Y, Toyomizu M. Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle. Am J Physiol Regul Integr Comp Physiol 297: R690–R698, 2009. First published June 24, 2009; doi:10.1152/ajpregu.90974.2008.—We have previously shown that avian uncoupling protein (avUCP) is downregulated on exposure to acute heat stress, stimulating mitochondrial reactive oxygen species (ROS) production and oxidative damage. In this study, we investigated whether upregulation of avUCP could attenuate oxidative damage caused by acute heat stress. Broiler chickens (Gallus gallus) were fed either a control diet or an olive oil-supplemented diet (6.7%), which has been shown to increase the expression of UCP3 in mammals, for 8 days and then exposed either to heat stress (34°C, 12 h) or kept at a thermoneutral temperature (25°C). Skeletal muscle mitochondrial ROS (measured as H2O2) production, avUCP expression, oxidative damage, mitochondrial membrane potential, and oxygen consumption were studied. We confirmed that heat stress increased mitochondrial ROS production and malondialdehyde levels and decreased the amount of avUCP. As expected, feeding birds an olive oil-supplemented diet increased the expression of avUCP in skeletal muscle mitochondria and decreased ROS production and oxidative damage. Studies on mitochondrial function showed that heat stress increased membrane potential in state 4, which was reversed by feeding birds an olive oil-supplemented diet, although no differences in basal proton leak were observed between control and heat-stressed groups. These results show that under heat stress, mitochondrial ROS production and oxidative damage of ROS production may occur due to changes in respiratory chain activity as well as avUCP expression in skeletal muscle mitochondria.

mitochondria; reactive oxygen species; membrane potential; uncoupling protein; proton leak

THE PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) occurs to a large extent in mitochondria (11): when electron flow through the respiratory chain is elevated, “backed up” electrons may react with oxygen to produce ROS. It is well-known that ROS production can be decreased by mild uncoupling of mitochondrial respiration (6, 48). Uncoupling proteins (UCPs) are specialized members of the mitochondrial transporter family that allow passive proton transport through the mitochondrial inner membrane. This transport activity leads to uncoupling of mitochondrial respiration and to energy waste, which is well documented with UCP1 in brown adipose tissue. The uncoupling activity of more recently discovered UCPs (post-1997), such as UCP2 and UCP3 in mammals or avian UCP (avUCP) in birds, is more difficult to characterize. However, recent extensive data support the idea that the newly discovered UCPs are involved in the control of ROS generation rather than thermogenesis (1, 4, 16, 34). This fits with the hypothesis that mild uncoupling caused by the UCPs decreases ROS production.

ROS production by the electron transport chain is favored by high cellular oxygen content and/or a highly reduced state of the electron transport chain. Mechanisms that reduce membrane potential could therefore reduce ROS production. Skulachev (47) suggested that even a small decrease in mitochondrial proton motive force could significantly lower ROS production by mitochondria. This suggestion was experimentally verified (24, 27), and it was assumed that such “mild” uncoupling mediated by UCP2 and UCP3 may serve as an antioxidant mechanism (48). Knocking out UCP2 or UCP3 resulted in a particularly strong prooxidant effect, whereas the overproduction of ROS greatly increased the antioxidant capacity of the cell (4, 26, 34, 52). In fact, Vidal-Puig et al. (52) reported an increase in the ROS level in skeletal muscles from UCP3 knockout mice. Brand et al. (7) reported that UCP3 knockout mice showed signs of increased oxidative damage to skeletal muscle mitochondrial proteins. All these effects seem to indicate the involvement of UCP2 and UCP3 in the cell antioxidant response (3).

Heat stress is an environmental factor responsible for stimulating ROS production. Our group (32) has already provided direct evidence of mitochondrial superoxide generation using both electron spin resonance spectroscopy, with 5,5-dimethyl-1-pyrroline N-oxide as a spin trap agent, and lucigenin-derived chemiluminescence in skeletal muscle of acute heat-stressed birds. It was also shown that acute heat treatments caused oxidative damage to mitochondrial proteins and lipids in skeletal muscle of birds (30). Under heat stress conditions, we have shown that downregulation of avUCP protein and mRNA expression was accompanied by increased mitochondrial superoxide production (31) and that these effects occurred in a time-dependent manner (29). Therefore, it can be assumed that avUCP, expressed appropriately, may play a role in the alleviation of mitochondrial ROS production and an antioxidant role under conditions of acute heat stress.

In this study, we hypothesized that upregulation of avUCP mainly attenuates mitochondrial ROS production and oxidative damage in birds exposed to acute heat stress. To upregulate avUCP expression, we fed dietary olive oil, which has been shown to increase the expression of UCP3 in mammals (40), to birds. The high levels of oleic acid in the dietary olive oil may activate the expression of UCP3 mRNA (22). We have investigated another possible manner in which, on exposure to heat stress, changes in mitochondrial ROS production by heat stress or olive oil supplementation in diet depend on membrane potential. The change in mitochondrial ROS production, which is independent of the levels of avUCP in skeletal muscle mitochondria, could be controlled by respiratory chain activity.

Address for reprint requests and other correspondence: M. Toyomizu, Nutritional Biochemistry of Animals, Life Sciences, Graduate School of Agricultural Science, Tohoku Univ., 1-1 Tsutsuamidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan (e-mail: toyomizu@bios.tohoku.ac.jp).
OLIVE OIL ALLEVIATES ACUTE HEAT STRESS-INDUCED ROS

**MATERIALS AND METHODS**

*Animals and experimental design.* Two experiments were conducted. The first experiment studied the effect of olive oil on alleviation of heat stress-induced skeletal muscle mitochondrial ROS (measured as H$_2$O$_2$) production and oxidative damage by changes in avUCP expression. In the second experiment, mitochondrial membrane potential and oxygen consumption in states 3 and 4 were measured in chicks fed olive oil and exposed to acute heat stress.

Meat-type male chicks (Cobb) were obtained from a commercial hatchery (Economic Federation of Agricultural Cooperatives, Iwate, Japan) at 1 day of age. The chicks were housed in electrically heated batteries under continuous light for 6 days and provided with ad libitum access to water and commercial starter meat-type chick diet (crude protein, 22%; metabolizable energy content, 3,000 kcal/kg). After a 2-day adaptation period, two kinds of diet, i.e., commercial diet and olive oil-supplemented diet (6.7 parts of olive oil were additionally supplemented in 100 parts of basal diet), were fed to two groups each for 8 days. One of the two groups was then exposed to 34°C for 12 h, while the other group was maintained at 25°C (humidity 55 ± 5%). The experimental diets were stored at −80°C until required for extraction of total RNA. After heat exposure following dietary treatment, eight birds in each group were selected for preparation of four samples of mitochondria isolated from two individual pooled muscles, and six of them were used for biochemical analysis of muscle samples. We used the pectoralis muscle for our study because avUCP is predominantly expressed in skeletal muscle of chickens (36, 51). To study the expression of genes, muscles were frozen, powdered in liquid nitrogen, and stored at −80°C until required for extraction of total RNA. A sample of muscle was placed in ice-cold isolation medium (100 mM KCl, 50 mM Tris·HCl, and 2 mM EGTA, pH 7.4) for mitochondria isolation (described below). The Animal Care and Use Committee of the Graduate School of Agricultural Science of Tohoku University approved all procedures in this experiment, and efforts were made to minimize pain or discomfort of the animals.

*Isolation of mitochondria.* After the birds were killed, skeletal muscle (pectoralis superficialis) was immediately dissected from the chicken breast for mitochondrial isolation by homogenization, protein digestion, and differential centrifugation according to the standard method (10). All procedures were carried out at 4°C. Muscle was trimmed of fat and connective tissue, blotted dry, weighed, and placed in isolation medium on ice. Tissue was shredded and minced with sharp scissors, rinsed with isolation medium three times, stirred for 5 min in protein digestion medium [100 mM KCl, 50 mM Tris·HCl, and 2 mM EGTA, 1 mM ATP, 5 mM MgCl$_2$, 0.5% (wt/vol) bovine serum albumin (BSA), and 11.8 units of protease per gram of tissue (Sigma subtilisin type VIH), pH 7.4], and then gently homogenized using a Polytron tissue homogenizer. The homogenate was stirred for 6 min and then mixed with the equivalent medium without proteinase to stop protease activity. The homogenate was then rehomogenized with a Potter-Elvehjem homogenizer (5 passes) and centrifuged at 1,000 g for 10 min. The supernatant was filtered through muslin and centrifuged at 10,400 g for 10 min. Mitochondrial pellets were resuspended in isolation medium and centrifuged at 10,400 g for 10 min, then at 3,800 g for 10 min, and were resuspended in isolation medium. Protein concentration was determined by the bicinchoninic acid assay with BSA as the standard (9).

*Mitochondrial H$_2$O$_2$ production.* H$_2$O$_2$ generation rates were determined fluorometrically by measurement of oxidation of p-hydroxyphenyl acetic acid (PHPA) coupled to the enzymatic reduction of H$_2$O$_2$ by horseradish peroxidase (17). Muscle mitochondria (0.35 mg/ml) were incubated in standard incubation buffer [120 mM KCl, 5 mM KH$_2$PO$_4$, 3 mM HEPES, 1 mM EGTA, and 0.3% (wt/vol) BSA, pH 7.2, 37°C] containing 50 μg/ml PHPA, 4 U/ml horseradish peroxidase, and 30 U/ml superoxide dismutase. After the addition of 4 mM succinate, the H$_2$O$_2$ generation rates were determined spectrophotometrically as the change in fluorescence at excitation and emission wavelengths of 320 and 400 nm, respectively. The assay was carried out on a computer-controlled spectrophotometer with appropriate correction for background and use of a standard curve.

*Quantification of mRNA using real-time RT-PCR.* Standard molecular biological techniques were used, essentially as described by Sambrook et al. (44). Tissues were homogenized in Trizol reagent (Invitrogen, San Diego, CA), and total RNA was isolated according to the manufacturer’s protocol. To study alterations in the expression of skeletal muscle target genes avUCP and avian adenine nucleotide translocator (avANT), we performed real-time RT-PCR analysis using the iCycler real-time detection system (Bio-Rad Laboratories, Hercules, CA). Five micrograms of total RNA were reverse transcribed using a mixture of oligo(dT)$_{12-18}$, random primers, and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Oligonucleotide primers and melting curve profiles were recorded. Oligonucleotide sequences of sense and antisense primers and annealing temperatures were the same as previously described (29). The specificity of the amplification product was further verified by electrophoresis on a 0.8% agarose gel and by DNA sequencing. Results are presented as the ratio of mRNA to 18s rRNA to correct for differences in the amounts of template cDNA used.

*Quantification of avUCP protein using Western blot analysis.* Western blot analysis for avUCP in mitochondria isolated from the pectoralis muscle was carried out as described previously (1, 29). Mitochondrial oxygen consumption and membrane potential. Mitochondrial oxygen consumption and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylyl phosphonium cation (TPMP$^+$, respectively (10, 18). Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) maintained at 38°C and calibrated with an air-saturated assay medium [120 mM KCl, 5 mM KH$_2$PO$_4$, 3 mM HEPES, 1 mM EGTA, and 0.3% (wt/vol) BSA, pH 7.2, 38°C] that was assumed to contain 402 nmol of oxygen per milliliter (39). The TPMP$^+$ electrode was calibrated with sequential additions of 0.5 up to 2 μM TPMP. Electrode linearity was routinely checked by following the uncoupled respiration rate in the presence of 0.71 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from 100 to 0% air saturation and to dissipate the membrane potential and release all TPMP back into the medium, allowing correction for any small electrode drift. Mitochondria (0.25 mg protein/ml) were incubated in assay medium containing 5 μM rotenone, and 4 mM succinate was added to start the reaction. The TPMP$^+$ binding correction for skeletal muscle was taken to be 0.45 micromolar per milligram of protein (41). The kinetics of proton conductance were measured in nonphosphorylating mitochondria to avoid interference by any changes in the rate of respiration driving phosphorylation (17). Mitochondria from skeletal muscle (0.35 mg/ml) were incubated in the above assay medium with 5 μM rotenone, 80 ng/ml nigericin, and 1 μg/ml oligomycin. Respiration rate and membrane potential were measured simultaneously after 4 mM succinate was added as substrate following sequential 0.5 μM additions of TPMP$^+$ up to 2 μM TPMP. Membrane potential was varied by adding malonate (up to 3 mM). After each run, 0.71 μM CCCP was added to release TPMP$^+$ for baseline correction. The entire proton leak kinetic measurements required about 10 min.

*Mitochondrial and muscle malondialdehyde.* Mitochondrial and muscle lipid peroxidation were determined colorimetrically as a
2-thiobarbituric acid-reactive substance, as described previously (30). The samples were stored at −80°C and analyzed within 1 wk.

Statistical analysis. Data were analyzed using the SAS statistical program package (SAS Institute, Cary, NC). Differences between control diet-fed and olive oil-fed groups were assessed using Student’s t-test for unpaired data. All other data were first analyzed using a general linear model analysis of variance, whereas means were compared using Duncan’s least significance multiple-range test. All data are means ± SE. Differences were considered significant for values of P < 0.05.

RESULTS

Growth performance and feed consumption. Feeding chickens a diet containing olive oil for 8 days under thermoneutral conditions significantly increased the weight gain of birds compared with that of control diet-fed birds (Fig. 1A), whereas no change was observed in feed intake.

On exposure to heat stress for 12 h, growth performance and feed consumption significantly decreased in birds maintained on a control diet (Fig. 1B). This decrease in production performance due to heat stress was limited to some extent in birds fed a diet containing olive oil.

Mitochondrial H2O2 production. Under thermoneutral conditions, birds fed a diet containing olive oil showed lower mitochondrial H2O2 production compared with control diet-fed birds (Fig. 2). On exposure to heat stress, H2O2 production was significantly increased in mitochondria isolated from control diet-fed birds. Heat-stressed birds fed dietary olive oil showed decreased mitochondrial H2O2 production compared with control diet-fed heat-stressed birds. Importantly, the olive oil-fed heat-stressed birds’ mitochondrial H2O2 production was similar to that of basal control levels in birds not exposed to heat stress.

Avian UCP and avian ANT gene expression and avian UCP protein content. Birds fed a diet containing olive oil under thermoneutral conditions showed an upregulation of avUCP gene transcripts in skeletal muscle (Fig. 3A) and an increase in avUCP protein content in muscle mitochondria (Fig. 3B). On exposure to heat stress, skeletal muscle avUCP mRNA and avUCP protein content in mitochondria decreased in control diet-fed birds. These decreases in avUCP gene expression and avUCP mitochondrial protein content on exposure to heat stress recovered to baseline levels in olive oil diet-fed birds. AvANT gene expression was not changed by either a diet containing olive oil or exposure to heat stress (Fig. 3C).
Mitochondrial oxygen consumption and membrane potential. Oxygen consumption and membrane potential were measured in mitochondria isolated from pectoralis muscle of control birds and heat-stressed birds fed either a control diet or an olive oil-containing diet (Fig. 4A). Succinate was used as a substrate to energize the mitochondria. Membrane potentials in state 4 of muscle mitochondria from heat-stressed birds on a control diet were appreciably higher than those for thermoneutral control or olive oil-fed birds and heat-stressed birds fed olive oil-supplemented diets. Membrane potential in state 3 and oxygen consumption in state 3 and state 4 for heat-stressed birds fed the control diet were even slightly higher than other groups. Their values in heat-stressed birds fed a diet containing olive oil were comparable to those obtained for the thermoneutral control or olive oil-fed group: both membrane potential and oxygen consumption in states 3 and 4 were similar among the thermoneutral control and olive oil-fed groups and the heat-stressed group fed a diet containing olive oil except the reducing effect of olive oil supplementation on mitochondrial membrane potential in state 4 of thermoneutral groups, which was not marked, but definite. Figure 4C shows the proton leak kinetics of muscle mitochondria for the thermoneutral control and olive oil-fed groups and the heat-stressed groups fed control and olive oil diets. As shown in Fig. 4C, basal proton leaks were similar to one another at any membrane potential, but the heat-stressed birds on a control diet exhibited the highest values of mitochondrial membrane potential and oxygen consumption on the right in the kinetics curves (state 4) compared with those for other groups.

Oxidative damage to mitochondria and muscle. Birds fed an olive oil-supplemented diet under thermoneutral conditions exhibited lower mitochondrial and muscle malondialdehyde (MDA) levels compared with control diet-fed birds (Fig. 5). On exposure to heat stress, the mitochondrial and muscle MDA levels were significantly increased in control diet-fed birds. Olive oil-fed heat-stressed birds showed increased mitochondrial and muscle MDA levels compared with thermoneutral olive oil-fed birds, but these levels were less than those reached in control diet-fed heat-stressed birds.

DISCUSSION

Birds fed an olive oil-supplemented diet showed better production performance compared with control diet-fed birds, whether under thermoneutral conditions or when exposed to heat stress. On exposure to heat stress, the control diet-fed birds showed marked body weight loss, whereas in olive oil-fed birds, the negative effects of heat stress on growth were reduced. It was clearly shown that under thermoneutral or heat stress conditions, the birds fed a diet containing olive oil produced lower levels of “mitochondrial ROS” compared with control diet-fed birds. This lowered ROS production may have lessened the decrease observed in body weights of heat-stressed birds fed an olive oil diet compared with controls. In the present study, mitochondrial ROS generation in the skeletal muscle of birds was increased on exposure of the animals to heat stress, and this increase possibly decreased the production performance of the birds. In contrast, feeding the birds an olive oil-supplemented diet decreased mitochondrial ROS production, thereby contributing to better production performance.

One reason olive oil-fed birds are able to reduce mitochondrial ROS production may be due to the upregulation of avUCP. UCPI in thymus (12), as well as other low-abundance UCPs (UCP2 to UCP5), is able to attenuate mitochondrial ROS production (1, 4, 16, 34, 43). In our previous studies in heat-stressed birds whose mitochondrial avUCP gene expression and protein levels were downregulated, skeletal muscle mitochondria-derived ROS production was increased compared with control birds (29, 31). These findings allow us to hypothesize that avUCP may play a key regulatory role in limiting mitochondrial ROS produced under heat stress conditions. To confirm this hypothesis, we studied avUCP gene expression in muscle and immunodetected the avUCP protein content in muscle mitochondria of birds kept under thermoneutral or heat stress conditions and fed either a control or olive oil-supplemented diet. In the present study, we have shown that dietary olive oil upregulates skeletal muscle avUCP gene expression and mitochondrial protein content under thermoneutral conditions. Rodriguez et al. (40) compared the influence of four dietary lipid sources (i.e., olive oil, sunflower oil, palm oil, and beef tallow) on UCP1, UCP2, and UCP3 contents and the levels of mRNA expression of these three proteins in
several tissues of the rat. Olive oil feeding increased UCP1, UCP2, and UCP3 mRNA expression in interscapular brown adipose tissue. An analogous effect also was observed in gastrocnemius muscle UCP3 mRNA expression levels. The findings of the present study are in agreement with the previous report, since feeding an olive oil-supplemented diet resulted in increased avUCP gene expression in the skeletal muscle of birds (Fig. 3A). Heat stress resulted in a 70% reduction of avUCP gene transcripts in control diet-fed birds. On the other hand, in olive oil diet-fed heat-stressed birds, avUCP gene transcript levels were similar to those levels in control birds kept at thermoneutral temperature. To confirm the expression of avUCP mRNA in olive oil diet-fed heat-stressed birds at the protein level, we analyzed mitochondrial avUCP protein content using Western blotting, since an increase in mRNA expression in general is not always reflected in the expression of a protein itself (29, 35, 40). The results of the present study showed that the immunodetected mitochondrial avUCP protein content in olive oil diet-fed heat-stressed birds was similar to levels in thermoneutral olive oil diet-fed birds and was higher than that of control diet-fed heat-stressed birds. Thus not only the skeletal muscle gene transcripts but also the mitochondrial avUCP protein contents were upregulated by adding olive oil to the birds’ diet. This enhancement of the avUCP protein in mitochondria may contribute to the reduced mitochondrial ROS production in heat-stressed birds, possibly via an increased inducible proton leak that would accompany the reduced membrane potential in mitochondria. To this extent, mild uncoupling resulting in a small decrease in membrane potential has been suggested to have a natural antioxidant effect (46).

It is well established that activation of UCP-mediated uncoupling would attenuate mitochondrial ROS production and limit ROS-induced cellular damage. Considering the suggestions of Skulachev (48) and Brand et al. (7) that elevation of mitochondrial membrane potential due to defects in UCPs could be a cause of increased superoxide production and that UCP-mediated mild uncoupling could be sufficient to suppress mitochondrial superoxide production, upregulation of avUCP in heat-stressed birds fed dietary olive oil may have lowered the heat-stress-induced increases in mitochondrial membrane potential, resulting in lower ROS production. This notion is supported by the recent demonstration by Dlaskova et al. (15) that any effect leading to more coupled states leads to enhanced...
ROS generation, whereas any effect resulting in uncoupling gives reduced ROS generation in brown adipose tissue mitochondria. In the present study, we did not access the capacity of avUCP to mediate proton leak: although maximal UCP stimulation can be obtained by a supraphysiological dose of artificial exogenous superoxide, which is generated by the enzymatic reaction of xanthine and xanthine oxidase (XXO), to estimate UCP activity for the evaluation of uncoupling-induced proton leak (38, 50), the XXO may also affect mitochondrial redox status and/or membrane integrity and may interfere with oxygen electrode measurements (14). Further experimental and comparative approaches are needed to demonstrate that avUCP could mediate the mild uncoupling able to reduce ROS.

The findings that not only UCP but also ANT can mediate uncoupling by free fatty acids (2, 45, 50) allows us to postulate that the increased ROS production in heat-stressed birds might also be associated with the suppression of ANT. It was shown that knocking out one of two ANT isoenzymes (muscle-specific ANT1) resulted in a strong increase in ROS production by muscle mitochondria (19). Recently, our group (29) found that heat stress did not affect avANT transcript expression. In the present study, avANT expression was not changed in birds fed either an olive oil diet or a control diet under thermoneutral conditions or exposed to heat stress. Thus avANT and avUCP gene transcripts and mitochondrial anion transporters involved in oxidative phosphorylation seem to be regulated differently. On exposure to heat stress, avUCP gene expression was downregulated, whereas avANT expression remained unchanged (Fig. 3, A–C). The upregulation of avUCP by feeding birds an olive oil-supplemented diet led to the expression of this protein, which possibly plays a role in reducing mitochondrial ROS production in olive oil diet-fed heat-stressed birds. AvANT, on the other hand, does not seem to play a significant role under these conditions.

To further characterize the mitochondrial function, we studied mitochondrial oxygen consumption and membrane potential in state 3 (in the presence of ADP) and state 4 (in the absence of ADP) in control and heat-stressed birds fed a control or olive oil-supplemented diet. Miwa and Brand (28) reported that there is a strong positive correlation between membrane potential and ROS production in isolated Drosophila mitochondria. It also has been emphasized that ROS generation shows a very steep dependence on the membrane potential (ΔΨ) value. The slight decrease in ΔΨ caused significant inhibition of the ROS production (49). Therefore, one can suppose that mitochondrial overproduction of ROS would result from an increase in mitochondrial membrane potential. Results of the present study show that in muscle mitochondria of heat-stressed birds, the membrane potential was higher in state 4 compared with that of control birds (Fig. 4A). On the other hand, in mitochondria isolated from olive oil diet-fed heat-stressed birds, membrane potential in state 4 was lower than those of control diet-fed heat-stressed birds, although the difference was not significant between the thermonutral control and olive-fed groups. Thus it is conceivable that the mitochondrial membrane potential might be responsible for ROS production of skeletal muscle. Importantly, as described by Rolfe et al. (41), the control of state 4 respiration (Fig. 4B, point a) is shared between “respiratory chain” activity (Fig. 4B, line c) and “basal proton leak kinetics” (Fig. 4B, line b); In state 4, an increase in membrane potential (Fig. 4B, point a’ or point a’’) could be due to a reduced proton leak (Fig. 4B, line b’ or to an enhanced activity of respiratory chain (Fig. 4B, line c’). Data presented in Fig. 4A clearly show that heat stress induces an increase in mitochondrial membrane potential and respiration rate in state 4 compared with control animals. On the other hand, the administration of olive oil to heat-stressed animals restored mitochondrial membrane potential and respiration rate to control values when both state 4 and state 3 were considered. From these findings we could hypothesize that in state 4, an increase in membrane potential in the muscle mitochondria of acute heat-stressed birds compared with that of the thermonutral control ones might be due to an increase in respiratory chain activity and that this activity could be inhibited in olive oil-fed heat-stressed animals, thereby reducing membrane potential. This hypothesis was supported by the results of Fig. 4C, which show that the basal proton leaks of muscle mitochondria from four treatment groups are similar to one another at any membrane potential, although they are slightly lower in the olive oil diet group than in the control diet group. The heat-stressed birds on a control diet exhibited the highest values of mitochondrial membrane potential and oxygen consumption as shown at the right in the kinetics curves (state 4) compared with those for other groups. From these results, it is very likely that the variations in membrane potential formation associated with heat stress or dietary olive oil treatment are the result of variations in the activity of respiratory chain and not the basal proton leak or the amount of avUCP, which should be associated with an inducible proton leak. In this way, the mitochondrial membrane potential was varied in olive oil-fed heat-stressed animals, thereby influen-
PUFA alter the mole percent of linoleic acid plus arachidonic acid in cardiolipin of colonocyte mitochondrial membrane, leading to changes in ROS production (13). Therefore, one may speculate that as PUFA was consumed to relatively low levels in the olive oil-supplemented group, ROS production decreased in mitochondria. In our present study, however, the amount of basal feed consumed by the control group and olive oil-fed group was 454 and 427 g [456 ± 100/(100 + 6.7)] for 8 days, respectively, suggesting that the ratio of amount consumed of PUFA or other components for control birds to that for olive oil-fed ones is 100/94. Moreover, Lemieux et al. (25) recently showed that feeding 8% olive oil to rats had no effect on the PUFA content of heart mitochondrial membrane compared with that of rats fed coconut oil, which represents an enriched source of saturated fatty acids. Therefore, feeding of olive oil-supplemented diets may not exert serious effects of PUFA or others on mitochondrial ROS production. On the other hand, there could be a dietary caloric effect on ROS production, because the energy density of olive oil-supplemented diet is ≥1.2 times that of the control diet even though the total amounts of feed intake were very similar. However, decreased ROS production of olive oil fed birds in this study is probably not due to increases in calorie intake, since it is known that the decrease in mitochondrial ROS generation and oxidative damage to mtDNA occurs during dietary restriction (21). Taken together, other factors besides olive oil may not necessarily need to be considered. There also could be possible effects of polyphenols in olive oil, which can provide benefits for plasma lipid levels and oxidative damage (37), if a virgin olive oil with high natural phenolic content was used in this study. However, this is not the case, because refined olive oil was used in our study. Refined olive oil has a low phenolic content, since these compounds are lost in the refinement process.

**Perspectives and Significance**

Heat stress is an environmental factor responsible for oxidative stress and damage that has great implications for animal production as well as human life. Our present study provides a new idea for dietary manipulation in which an olive oil-supplemented diet is shown to control ROS production and oxidative damage under thermoneutral or acute heat stress conditions. The effect of olive oil-supplemented diets on ROS production is possibly due to its effect on membrane potentials via UCP-mediated proton leak or respiratory chain activities in skeletal muscle mitochondria. This study demonstrates the use of birds as possible models in experiments on nutritional regulation of gene expression, mitochondrial ROS production, and bioenergetics. Results have significance to research in mitochondrial bioenergetics, stress-nutrient-gene interactions, and oxidative stress and damage studies. Given that skeletal muscle tissue in chickens, as in large mammals including humans, plays an important role in overall bioenergetics and homeostasis, the current results obtained from chicken are likely to find application in research on large mammals and general health.

In conclusion, feeding diet oil reduces mitochondrial ROS production and lowers oxidative damage in birds under thermoneutral or heat stress conditions. Mitochondrial oxidative studies and avUCP expression analyses suggest that, under heat stress conditions, mitochondrial ROS production
and olive oil-induced downregulation of this ROS production may occur via changes not only in avUCP expression but also to altered respiratory chain activity in skeletal muscle mitochondria.

ACKNOWLEDGMENTS

We thank Dr. R. Smith, Jr., Tohoku University, for careful proofreading of this manuscript.

GRANTS

This work was supported by a grant for scientific research from the Ministry of Education, Science, and Culture of Japan (Grant-in-Aid 18380157).

REFERENCES


