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Nutritional level and energetic source are determinants of elevated circulatory lipohydroperoxide concentration

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Dietary energetic impact on oxidative stress is incompletely understood. Therefore, effects of diets on oxidative stress were studied using a cross-over block design. In Expt 1, intake of metabolizable energy (ME) was restricted or *ad libitum*. In Expt 2, isoenergetic and isonitrogenic diets were fed, replacing carbohydrate energy by energy of fatty acids. Circulatory lipohydroperoxides (LOOH), markers of acute oxidative stress, were expressed absolutely and in terms of cholesterol or TAG levels. In Expt 1, plasma (jugularis vein) LOOH was assayed in combination with whole-body oxidative metabolism using gas exchange and heart rate (HR) during feeding periods and at rest. In Expt 2, LOOH was assayed in plasma from portal and a large udder vein and a mesenteric artery. In Expt 1, intake increased V_{O2}, HR and LOOH following overnight fast with higher values ($P<0{\cdot}05$) when feeding ME *ad libitum*. Intake of ME *ad libitum* (3 weeks) increased cardiac protein of cytochrome oxidase and endothelial-type nitric oxide synthase ($P<0{\cdot}05$), indicating adaptation of the heart to higher activity. Transient HR responses evoked by an antidiabetic drug (levcromakalim) revealed a linear positive correlation with relative LOOH (r^2 0·79), supporting the relationship between oxidative metabolic rate and lipoperoxidation. Evidence for exogenous lipids as LOOH source provided the vessel-specific rise in LOOH through replacing carbohydrate ME by lipid ME (Expt 2). Thus, dietary energy level and energetic source are important for circulatory LOOH with a role of vascular activity in production of oxidant.

Acute oxidative stress: Energy-dense diet: Lipid-enriched diet

Lipid peroxidation has been described as an index of oxidative stress frequently evident in response to energy-dense diet[1]. Major targets of peroxidation are saturated and unsaturated long-chain fatty acids and several reactive oxygen metabolites have a potential to convert lipids to hydroperoxide-containing derivatives[2]. Taking into consideration the powerful antioxidant capacity of plasma[3], circulatory lipohydroperoxides (LOOH) are likely to occur in consequence of excessive oxidants in tissues capable of producing and transferring lipids into the circulation.

Mobilization of fatty acids and their transient increase in the bloodstream have been recently reported to occur during energy imbalance of lactating dairy cows[4]. This increase has been described to associate with a rise in oxidatively modified lipids[5] with changed bioactivity[6] while plasma antioxidant level remained virtually unchanged[7]. In this context, it is notable that saturated and unsaturated long-chain fatty acids have been used to compensate energy deficiency in dairy cows during early lactation, and unsaturated fatty acids have been used to alleviate fatty liver[8] and other disorders in lipid metabolism in rodents and man[9]. In turn, unsaturated fatty acids are readily oxidized either *via* auto-oxidation[10,11] or by reactive oxygen species[2]. Low efficacy of dietary antioxidant supplementation regarding plasma antioxidant capacity has been reported[12] and the biological activity of oxidized lipids, once formed, can not be easily abolished by antioxidants[13] although initial derivatives, including LOOH, are highly reactive and unstable[14,15]. Therefore, increase in circulatory LOOH can be considered as a marker of actually acute imbalance among generation of and protection from oxidants (acute oxidative stress).

Studies on the ageing process have suggested that long-term energetic restriction mitigates oxidative stress[16,17]. Data from short-term experiments[18] and from studies on the liver at the cellular level[19] are inconsistent with attenuating oxidative stress by energetic restriction. In addition, little information exists on whether oxidative stress measured *in vivo* or *ex vivo* refers to oxidative metabolic rate or dietary energetic source.

The purpose of this study was to determine the effect of feeding a meal that provides different levels of metabolizable energy (ME) on circulatory LOOH in relation to oxidative metabolic rate measured by gas exchange and heart rate (HR). We used a bovine model enabling repeated bleeding without serious impairment of animal's health to determine plasma LOOH concentration in combination with measurements of gas exchange and HR during the corresponding periods. To support the findings, we used an antidiabetic

Abbreviations: eNOS, endothelial-type nitric oxide synthase; HBS, HEPES-buffered saline; HR, heart rate; $K_{ATP/ADP}$, ATP/ADP-regulated potassium channels; LOOH, lipohydroperoxides; LSM, least square mean; ME, metabolizable energy; MER, metabolizable energy requirement at rest; MR, metabolic rate.

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drug to change HR. Evidence for the role of dietary long-chain fatty acids as targets of peroxidation was obtained by investigating the response of LOOH in plasma from several vessels to isoenergetic and isonitrogenic diets differing in the energetic source (carbohydrate-based diet *v.* diet enriched with long-chain fatty acids).

Experimental methods

Materials

Levcromakalim was a gift from SmithKline Beecham (UK). Teflon catheters were purchased from Braun (Melsungen, Germany) and the lipid hydroperoxide assay kit from Alexis Biochemicals (Grünberg, Germany). Organic solvents were from Roth (Karlsruhe, Germany), enzymes for the cholesterol assay from Serva (Heidelberg, Germany) and the antibodies from manufacturers as indicated. All other chemicals and biochemicals were bought from Sigma (Taufkirchen, Germany).

Experimental plan

Expt 1 was based on a crossover block design (Table 1). Eight young bulls (German Holstein dairy cattle), 10 months old and 270–280 kg in weight at beginning the experiments, were randomly assigned to two dietary groups (four animals per block, eight repetitions and thirty-two samples per time-point indicated). Diets were offered for 3 weeks (adaption period) followed by 1 week of measuring gas exchange, HR and bleeding on four successive days before and after feeding to time-points indicated. Blood was withdrawn via jugularis vein catheter. The indwelling Teflon catheters were installed 3 or 4 d before the start of bleeding. Possible inflammatory developments were monitored by measuring the rectal temperature and circulatory indices as described previously[20]. Drug-evoked change in HR was induced by administration of the antidiabetic drug levcromakalim, an opener of ATP/ADP-regulated potassium channels ($K_{ATP/ADP}$), via catheter at a dose of 80 nmol/kg body mass within 2 min. In experiments using glibenclamide, a $K_{ATP/ADP}$ inhibitor, this antidiabetic drug was administered (within a period of 2 min) 2 min before levcromakalim at a dose of 400 nmol/kg. These doses were used on the basis of a preliminary experiment indicating minimal-effective doses with animal care taken into account. Drugs were dissolved in dimethyl sulphoxide, diluting the solvent by aqueous 0·1 M-$NaHCO_3$ to 0·01 % (v/v).

Table 1. The balanced block design*

	Feeding level	
Period	Restricted	*Ad libitum*
1	A B C D	E F G H
2	E F G H	A B C D

*Letters denote individuals. Animals were assigned randomly to either a diet that offered 1.5 times (restricted) or 2.0 times (*ad libitum*) metabolizable energy requirement at rest (480 kJ/kg$^{0.75}$ d). They were used crossover, yielding four animals per block and eight per nutritional level with bleeding *via* jugularis vein catheter on four consecutive days (thirty-two samples per time-point indicated in the corresponding figure).

Expt 2 was based on a crossover design with three animals per block and twelve samples per vessel and day when not otherwise stated. Lactating Holstein cows, 4 years old and 750–790 kg in weight, were used to install chronic indwelling catheters in the portal vein, a cranial arterial mesenteric branch and a large udder vein. Occasionally, blood was withdrawn from the udder vein by puncture using vacutainer. Procedures for catheterization were similar to those described elsewhere[21,22]. Experiments began 3 weeks after surgery. Blood was sampled at 30 min intervals during a 3 h period beginning in the morning (09.45 hours) after feeding at 07.45 h on two consecutive days. Four samples subsequently to the first bleeding were used for assaying LOOH and lipids as described later. Catheterization and treatments were approved by the local Governmental Animal Care Advisory Committee.

Diets

In Expt 1, animals received a ruminant-adequate diet (time-points are shown in the figures) for 4 weeks. Diets provided 1.5 or 2.0 times ME requirement at rest (MER) with MER = 450 kJ/kg$^{0.75}$ d. MER has been defined by preliminary experiments.

The diet consisted of artificial dried grass (not pelleted, 4·4 kg per ration; pelleted, 2 kg per ration) and differential concentrate (3·0 and 6·5 kg) to generate the different ME levels of 1·5 (restricted ME intake) and 2.0 times (*ad libitum* ME intake) MER. The composition of the diets is shown in Table 2. *Ad libitum* and restricted intake allowed intense and moderate growth (about 1050 and 550 g daily). Animal had free access to tap water and consumption was recorded. Adaptation to diets lasted 3 weeks, followed by 1 week of measurements.

In Expt 2, animals received isoenergetic and isonitrogenic diets with divergent sources of ME (Table 3 and 4). They were used in crossover in feeding a carbohydrate (starch)-enriched diet followed by a fat-based diet in periods of 3 weeks. In the fat-based diet, ME from starch was replaced mainly by long-chain fatty acids (Table 4). Palmitic and oleic acids were supplemented because these long-chain fatty acids are most abundant in tissues. Therefore, replacement of carbohydrate energy mainly by these fatty acids approximates the physiological situation.

Measurement of metabolic rate

The metabolic rate (MR) was non-invasively measured by indirect calorimetry using four climatized (18°C) open-circuit

Table 2. Diet composition (Expt 1)

Component	Roughage (g/100 g DM*)	Concentrate† (g/100 g DM*)
Crude protein	14·2	13·5
Crude fat	2·3	3·0‡
Crude fibre	29·8	6·5
Crude ash	6·7	4·2

*Digestibility of DM was 0·7, DM provided net energy of 10·5 MJ/kg DM.
†Supplement (3 g/kg) contained minerals (g/kg: Ca, 190; P, 100; Na, 95; Mg, 30; Zn, 6; Mn, 5; Cu, 1·2; I, 0·08; Se, 0·06; Co, 0·03) and vitamins (IU/kg: A 600 000; D_3 81 000; g/kg: E 0·75).
‡Total fatty acids, g/100 g DM, 1·55. SFA, g/100 g total fatty acids, accounted for 23, where portion of palmitic acid (16 : 0) averaged 22. MUFA accounted for 19 (oleic acid (18 : 1(9)) averaged 17) and PUFA for 59 (linoleic acid (18 : 2(9, 12)) averaged 52 and linolenic (18 : 3(9, 12, 15)) averaged 4).

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Table 3. Composition of starch- and fat-based diets (Expt 2)

Component	Starch diet (g/100 g DM)	Fat diet (g/100 g DM)
Crude protein	16·9	17·5
Crude fat	2·38	7·22
Crude fibre	17·5	18·0
Starch	18·4	10·4
Crude ash	8·86	9·45

respiration chambers with video cameras for recording position changes. Gas exchange was determined at 10 min intervals over at least 24 h. Calculations of MR based on V_{O2} and production of CO_2 and CH_4 were as described previously[20]. Under conditions of an intake approximately 1.5 times MER, the BMR (after an overnight fasting period of 17–18 h) was found to be a function of V_{O2}[20] whereas in non-steady-state conditions induced by meal intake the RER changed as shown later. Therefore, V_{O2} and RER were used to demonstrate dietary effects.

Animals were familiarized with the respiration chamber 2 weeks before beginning measurements as described previously[20]. HR was also used for estimating MR because of the relatively slow gas exchange changes, inducing a delay in the exact recordings of rapid transient responses to drugs. Bovine HR values have been demonstrated to provide data which correlated with MR via calibration by indirect calorimetry under several physiological conditions[20]. HR was measured by a bio-tachometer and responses to treatments were analysed by records for 20 s at intervals of 20 s. Least square means (LSM) of beats per minute were calculated by a computer-aided SAS program to compress the data representative for periods.

Detection of lipohydroperoxides

Blood (EDTA to 8 mM, pH 7·3) was collected *via* jugularis vein catheter (Expt 1) at time-points corresponding to feeding/treatment periods and after 17–18 h post-feeding in the afternoon (fasting overnight). It was placed immediately on ice. After centrifugation (3000 ***g***, 10 min, 4°C) plasma was mixed with butylated hydroxytoluene (to 20 μM), sucrose (to 0·43 M) and 4-(2-aminoethyl)benzene sulphonylfluoride (to 150–200 μM) to inhibit artificial oxidations and deacylation of phospholipids and then was stored frozen (−20°C). Concentration of LOOH was determined in organic-phase lipid extracts by an assay using 13-hydroperoxyoctadecadienoic acid as standard and ferrous (Fe^{2+}) sulphate–ammonium thiocyanate as chromogen following the instruction of a kit (Cayman) with some modifications as described previously[5] to meet limited sample material. CV of intra- (*n* 12) and inter-assays (*n* 6) were 12·4 and 15 %. This assay directly determines hydroperoxides of both saturated and unsaturated lipids[14]. These products of peroxidation are reactive and unstable. Therefore, their quantification has been reported to be useful[15] to detect actual transient oxidants that overwhelm defence (oxidative stress).

Table 4. Ingredients of starch- and fat-based diets (Expt 2)

Ingredient	Starch diet (g/100 g DM*)	Fat diet (g/100 g DM*)
Concentrate mix	8·8	9·3
Maize silage	25·5	27·3
Grass silage	23·6	25·2
Grass pellets	9·3	10·0
Maize starch	9·2	–
Protected fat†	–	5·0
Extracted soyabean meal	6·3	–
Protected soyabeans	–	12·3
Urea	0·4	–
Lime	0·8	–
Sugar-beet pulp	14·9	9·4
Vitamin-mineral premix	1·2	1·4

* DM content of starch- and fat-based diet was 466 and 455 g/kg, providing net energy based on lipid equivalents (NEL) 6·9 and 7·4 MJ/kg DM and utilizable protein 156 and 164 g/kg DM, respectively.

† Hajenol (NEL, 20·2 MJ/kg DM) from Harles & Jentzsch GmbH (Uetersen, Germany), containing mainly (v/w) palmitic acid (16 : 0) (44 %), stearic acid (18 : 0) (5 %), oleic acid (18 : 1(9)) (40 %), and linoleic acid (18 : 2(9, 12)) (9·5 %) as calcium salts.

Preparation of lipoprotein

The density of antioxidant-, sucrose- and 4-(2-aminoethyl)-benzene sulphonylfluoride-containing plasma was raised (0·38 g KBr/ml) and an aliquot (4 ml) was overlaid with 0·15 M-NaCl (2 ml) to transfer chylomicrons and VLDL into the supernatant by ultracentrifugation (100 000 ***g***, 14 h, 10°C). The supernatant was removed and the infranatant diluted with one volume of 0·15 M-NaCl. LDL was precipitated by addition of a mixture that consisted of aqueous dextrane sulphate-400 (to 0·015 %, w/v) and $MgCl_2$ (to 135 mM). Following 3 min on ice, LDL was spun off (5000 ***g***, 5 min, 4°C) and dissolved in HEPES-buffered saline (HBS; 15 mM-HEPES, 140 mM-NaCl, 0·2 mM-EDTA, pH 7·4). LDL particles were protected by 4-(2-aminoethyl)benzene sulphonylfluoride (100 μM) and sucrose and stored at −80°C. HDL of the supernatant obtained after spinning off LDL were precipitated through an increase in dextrane sulphate-400 to 0·045 % (w/v). The precipitate was collected by centrifugation, dissolved in HBS, protected by sucrose and stored at −80°C.

Immunocytofluorimetry

Muscle specimens were sampled in Expt 1 from the right ventricle of the heart and stored frozen (liquid nitrogen, then at −80°C) until analysis. Single cells were prepared by digestion of tissue (30 ± 10 mg wet weight) with 0·2 % (w/v) collagenase (type VIII) using a mixture of one volume of phenol red-free RPMI 1640 medium and one volume of HBS (RPMI/HBS). Single cells were filtered off after a gentle stirring period (1 min, intervals of 2 min, total 15 min at 37°C) and digestion was repeated. Cells were spun off (500 ***g***, 5 min, 4°C) to remove collagenase. The sedimentary fraction was resuspended with RPMI/HBS containing 0·5 % (w/v) bovine serum albumin. Total counts and cell volume were measured by cell counter (MöLab, Germany). Cells were fixed and permeabilized by methanol (−20°C) using one volume of suspension (10^7 cells/ml) and ten volumes of methanol, spun off (200 ***g***, 5 min, 0°C), resuspended in PBS–0·2 % (w/v) bovine serum albumin and aliquots were plated on ninety-six-well microtitre plates (Greiner, Germany). Aliquots were incubated at room temperature with rabbit antibodies against cytochrome oxidase with subunit IV as epitope (Molecular

Probes), and against Cu/Zn superoxide dismutase (Alexis), endothelial-type nitric oxide synthase (eNOS; Sigma) and 3-nitrotyrosine (Sigma) in final dilutions recommended by the manufacturers. Unbound antibodies were washed out and bound antibodies stained by anti-species antibody–fluorescein isothiocyanate conjugates. After incubation (2 h, room temperature) cells were washed and fluorescence of single cells was measured by flow cytometry (Beckman-Coulter, Elite). The phenotype was determined with β-actin as a marker of cardiomyocytes. In cellular aliquots, immunofluorescence which was generated by rabbit anti-β-actin (Sigma) and anti-rabbit Ig-phycoerythrine (Sigma), indicated >85 % immunopositive cells. Controls were obtained by replacing the specific primary antibodies by unrelated serum. Single cell analysis of fluorescence by flow cytometry followed the protocol described previously[20,23]. Briefly, histograms were established on the basis of cell size and granularity. Single cells (β-actin-positive cardiomyocytes) were gated, then fluorescence intensity per cell was measured by flow cytometric channel number (number increases with raising fluorescence intensity of a single cell and parallels the specific immunoreactive amount of antigen per cell), recording 5000 counts.

Other methods

The protein content of lipoprotein fractions was determined by protein-dye binding[24]. TAG and cholesterol were enzymatically assayed using kits from Sigma. Cholesterol was also enzymatically measured by a described technique[25], reducing volumes and ingredients to one-tenth to measure the colour on ninety-six-well microtitre plates.

Statistical analysis

Results are presented as means and standard deviations or as LSM and their standard errors as indicated. Pair-wise comparisons and comparison *v.* control were accomplished by ANOVA and *post hoc* Newman–Keuls procedure or Dunnett's method. Dunn's test was used in combination with ANOVA on ranks. Significance of the difference between two means was tested by Student's *t* test. *P* values $<0{\cdot}05$ were considered statistically significant. ANOVA, tests and analysis of linear regression were performed using SAS statistical package and Sigma Stat of the Jandel Scientific Software (version 1.02 1994; Erkrath, Germany). Graphic presentations were accomplished by Sigma Plot of the Jandel Scientific program package and an internal program package of the flow cytometer (Beckman-Coulter, Elite).

Results

Intake of nutrients

In Expt 1, net intake was 1·9 times MER when 2·0 times MER was offered (considering dietary residuals) and termed *ad libitum* intake in the following text in comparison with complete intake of the diet restricted to 1.5 times MER.

In Expt 2, the mean daily dietary intake (*n* 6) of DM, energy, crude protein, utilizable protein and crude fibre was 16·9 (SEM 0·3) kg, 120 (SEM 1) MJ (net energy based on lipid equivalents), 2·9 (SEM 0·2) kg, 2·7 (SEM 0·2) kg and 3·0 (SEM 0·2) kg, respectively. Intake did not differ between animals fed diets with starch or lipids as the major source of ME (*t* tests, two-sided, $P>0{\cdot}2$). Intake of starch averaged 3·2 (SEM 0·2) and 1·6 (SEM 0·2) kg/d and daily intake of crude fat averaged 0·4 (SEM 0·05) and 1·2 (SEM 0·05) kg ($P=0{\cdot}01$) when a starch- and a fat-based diet were offered, respectively. The present data provided evidence for differences in the energetic source following dietary intake at comparable (isoenergetic and isonitrogenic) nutritional level.

Response of relative lipohydroperoxide level to dietary intake (Expt 1)

Circulatory LOOH concentrations (Fig. 1 (A)) were found to resemble the pattern of values expressed per mol plasma total cholesterol. Values were lower in a ME restricted intake than in an *ad libitum* intake after overnight fast (Fig. 1 (B), 08.00 hours values). Following meal intake in the morning, LOOH (Fig. 1 (A)) and LOOH/total cholesterol (Fig. 1 (B)) rose in both diets (09.00 *v.* 08.00 hours values). Decrease in the LOOH level relative to the total cholesterol concentration was observed during the period between morning and afternoon intake to the ratio being evident after overnight fast. The present result suggests clearance of LOOH relative to total cholesterol. Intake in the afternoon raised the LOOH/total cholesterol ratio similar to a morning intake (Fig. 1 (B), 15.00 *v.* 14.00 hours values) with the higher rate in a ME *ad libitum* intake. The temporal course of LOOH relative to LDL cholesterol (Fig. 1 (C)) or HDL cholesterol (data not shown) resembled the pattern rendered by the LOOH/total cholesterol ratio. However, decrease in LOOH/LDL cholesterol (Fig. 1 (C), 14.00 *v.* 09.00 hours values) yielded values which did not significantly differ between diets. The present result was also found if LOOH concentrations referred to the level of TAG (Fig. 1 (D), 14.00 *v.* 09.00 hours values). A substantial decrease in the LOOH/TAG ratio feeding ME *ad libitum* in the morning (Fig. 1 (D), 14.00 *v.* 09.00 hours values) contrasted the insignificant decline in LOOH/TAG ratio (Fig. 1 (D), 14.00 *v.* 09.00 hours values) with restricted ME intake in the morning.

The dietary differences in LOOH levels relative to concentrations of LDL cholesterol or TAG (Fig. 1 (C,D)) were most conspicuous, comparing the values subsequently to an intake in the afternoon and after overnight fast (15.00 *v.* 08.00 hours values). Neither LDL cholesterol (1·10 (SEM 0·05) mM), HDL cholesterol (3·45 (SEM 0·23) mM) nor TAG (0·11 (SEM 0·02) mM) significantly differed among diets at these times. Therefore, the data indicate different LOOH clearance.

Dietary effect on heart rate and lipohydroperoxides (Expt 1)

Basal relative LOOH values (08.00 hours) corresponded to significant differences in the basal HR (0.00–06.00 hours period) between the ME restricted and *ad libitum* groups (Fig. 2 (A)).

After feeding in the morning, HR dramatically rose (Fig. 2 (A), 08.00–09.00 hours period) similar to relative LOOH values (Fig. 1 (A,B)). The rate of increments differed between diets. Taking the 11.00–13.00 hours period as the post-feeding state in the morning, afternoon ME restricted feeding tended to evoke a higher HR than did feeding ME

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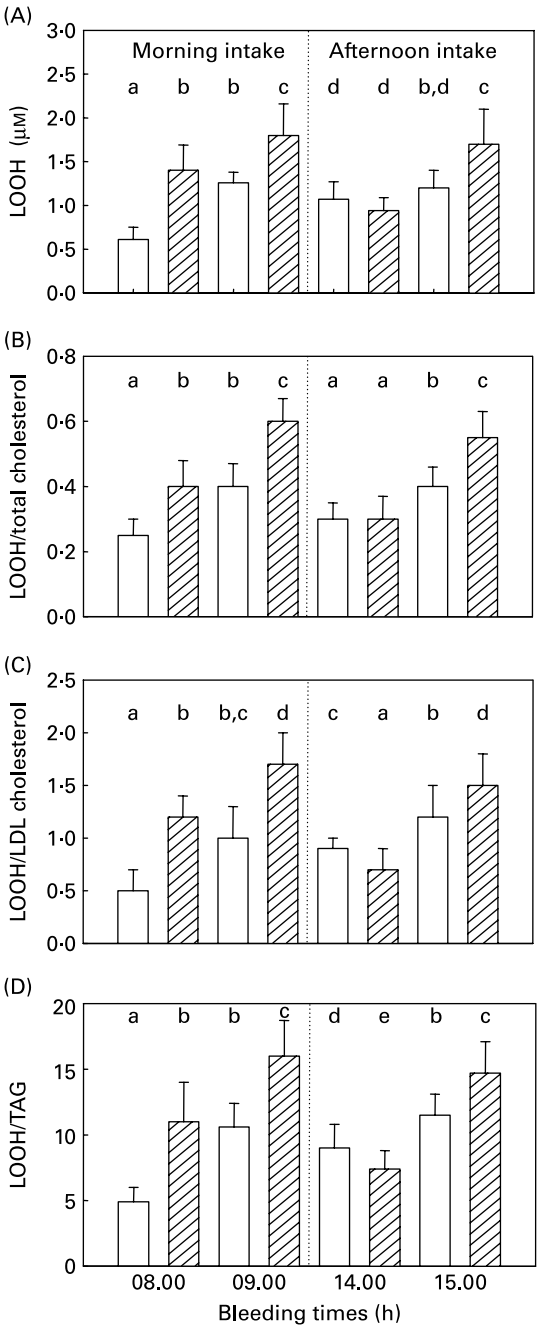

Fig. 1. Circulating lipohydroperoxide (LOOH) levels in response to different intakes of metabolizable energy (ME). (A), Temporal concentrations of plasma LOOH and those relative to total cholesterol (B), LDL cholesterol (C) and TAG (D) levels. For clarity, cholesterol values are multiplied by 10^{-3}. Values are means with standard deviations depicted by vertical bars (eight repetitions on four consecutive days, thirty-two samples per time-point), regarding *ad libitum* ME intake (▨) and restricted ME intake (□). Blood was drawn before and after food intake in the morning and afternoon as indicated. Plasma concentrations of LOOH, TAG and cholesterol were determined as described in the Experimental methods section. [a–d]Mean values with unlike superscript letters were significantly different (ANOVA, (A) $P=0{\cdot}0006$, (B) $P=0{\cdot}0008$, (C) $P=0{\cdot}0009$, (D) $P=0{\cdot}0005$; Newman–Keuls procedure, $P<0{\cdot}05$).

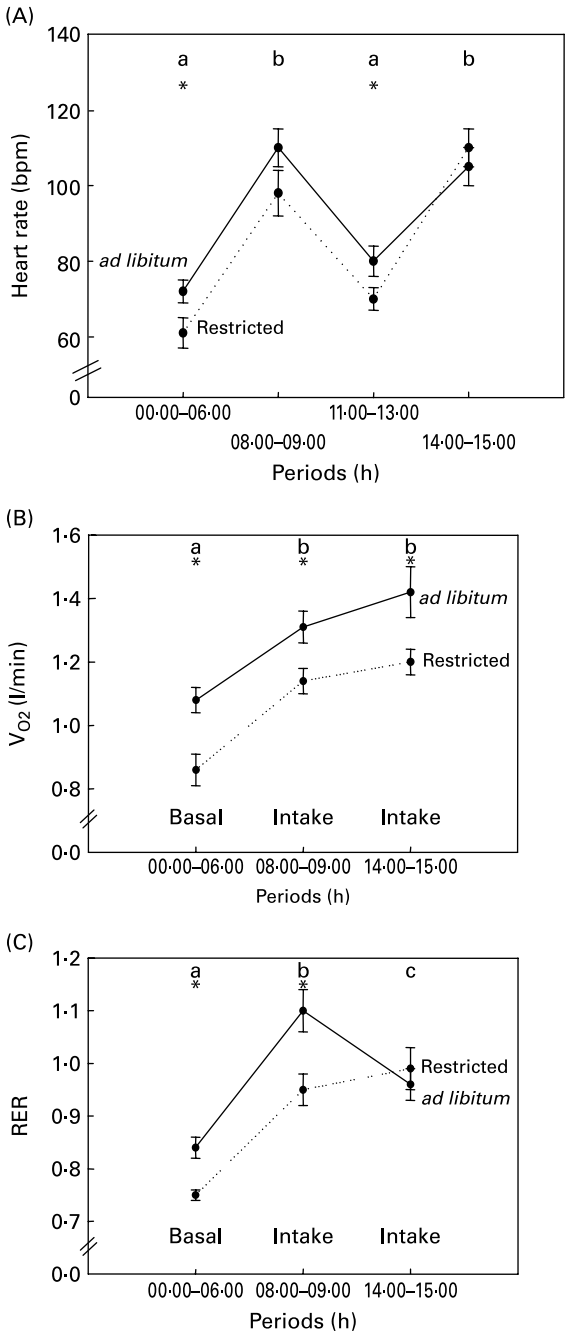

Fig. 2. Temporal course of intake-induced changes in gas exchange and heart rate. Heart rate, V_{O2} and production of CO_2 were continuously measured in respiration chambers. (A), Values are least square means (LSM), with their standard errors depicted by vertical bars, of the heart rate (beats per minute (bpm)) in periods corresponding with overnight fast (00.00–06.00 hours), morning meal intake (08.00–09.00 hours), intermediate rest (11.00–13.00 hours) and afternoon meal intake (14.00–15.00 hours). Mean values were significantly different between intake levels: ANOVA, $^{*}P=0{\cdot}0003$; Newman–Keuls procedure, $^{*}P<0{\cdot}05$ (n 8). [a,b]Temporal mean values with unlike superscript letters were significantly different (ANOVA, $P=0{\cdot}0005$; Newman-Keuls procedure, $P<0{\cdot}05$). V_{O2} data (B) and RER (C) are indicated in periods corresponding with overnight fasting (00.00–06.00 hours, basal) and *ad libitum* and restricted food intake. Food was offered in the morning and in the afternoon as indicated. Values are LSM with their standard errors depicted by vertical bars (n 8). Values were significantly different between intake levels: $^{*}P<0{\cdot}05$, Newman–Keuls procedure (n 8). [a,b,c]Temporal mean values with unlike superscript letters were significantly different (ANOVA, $P=0{\cdot}001$; Newman–Keuls procedure, $P<0{\cdot}05$).

ad libitum (Fig. 2 (A)). The present observation contrasted responses of relative LOOH in the afternoon, especially when LOOH levels were expressed in terms of total cholesterol concentration (Fig. 1 (A)), indicative of overlapping but not identical pathways, regulating basal LOOH and HR in comparison with intake-induced response.

Dietary effects on gas exchange (Expt 1)

Changes in V_{O2} (Fig. 2 (B)) and RER (Fig. 2 (C)) due to feeding after overnight fast paralleled the responses of HR (Fig. 2 (A)) and of relative LOOH levels (Fig. 1 (A–D)). An afternoon intake only tended to induce a further increase in V_{O2} in both diets (Fig. 2 (B)). The present response resembles that of relative LOOH level to a restricted intake, but contrasts the response of relative LOOH to a ME *ad libitum* intake. Taking into consideration RER (Fig. 2 (C)), higher intake of lipids *via* higher concentrate at the *ad libitum* level is strongly suggested to cause the divergent response, since preferred oxidation of lipids changes RER towards 0·7.

Relationship between lipohydroperoxides and drug-evoked heart rate (Expt 1)

The divergent responses of relative LOOH level and HR to a restricted and *ad libitum* intake led us to elicite a possible relationship between increase in HR and relative LOOH by treatment with levcromakalim. This drug has been reported to act as opener on $K_{ATP/ADP}$ at the neuronal and peripheral levels[26–30]. HR strongly rose (1.7 times the basal value) immediately post-levcromakalim (Fig. 3 (A)) administered into the jugularis vein. Glibenclamide, a $K_{ATP/ADP}$ inhibitor, attenuated the increase (Fig. 3 (A)), indicative of a specific $K_{ATP/ADP}$-mediated response. Afternoon feeding post-levcromakalim induced an attenuation of the HR (Fig. 3 (A)) as expected due to the known effect of the drug on cardiomyocytes[26,27]. Concentration of LOOH expressed in terms of total cholesterol virtually followed the temporal course of the HR induced by the $K_{ATP/ADP}$ opener (Fig. 3 (B)). Relative LOOH level post-glibenclamide did not significantly differ from values after treatment with the carrier of the drugs (for clarity the data were omitted from Fig. 3 (B)). Co-variation between HR and plasma LOOH was quantified by plotting the corresponding values shown in Fig. 3 (A) and Fig. 3 (B) and analysis of regression (Fig. 3 (C)). A virtually linear slope was obtained, explaining roughly 79 % of the total variance by the covariance between both variables (energetic intake *ad libitum*). Responses to the drugs of animals fed restricted ME were weaker with significant effects only up to 10 min following treatment (data not shown).

Dietary effect on abundance of cardiac proteins and nitrotyrosine (Expt 1)

The expression analysis focused on cardiomyocytes as striking temporal changes in relative circulatory LOOH concentration varied virtually on a parallel with HR. The portion of unspecific fluorescence overlapping specific fluorescence was low in cardiomyocytes (β-actin positive cells) as demonstrated for cytochrome oxidase (Fig. 4 (A)), indicative of specific

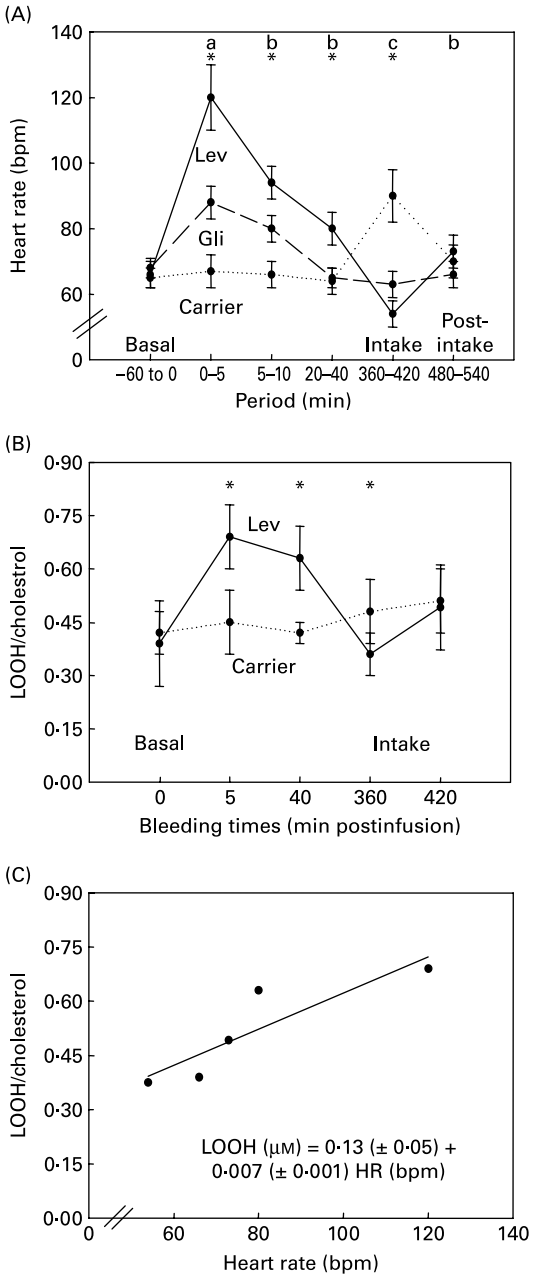

Fig. 3. Drug-evoked correlation between plasma lipohydroperoxide (LOOH) level and heart rate. (A), Values are least square means (LSM), with their standard errors depicted by vertical bars, of the heart rate (beats per minute (bpm)), using records in periods as indicated to demonstrate instant responses to treatment (5 min periods), temporal attenuation (20–40 min period) and modified responses to intake of a meal providing metabolizable energy *ad libitum*. Basal LSM refers to the period before treatment (−60 to 0 min). Levcromakalim (Lev, 80 nmol/kg, *n* 8) was administered as an opener, glibenclamide (Gli, 400 nmol/kg 2 min before Lev, *n* 4) as an inhibitor of ATP/ADP-regulated potassium channels. Mean values were significantly different between treatments with Lev and the carrier: ANOVA, $P=0{\cdot}002$; Newman–Keuls procedure, $P<0{\cdot}05$. ^{a,b,c}Temporal LSM of Lev values with unlike superscript letters were significantly different (ANOVA, $P=0{\cdot}0003$; Newman–Keuls procedure, $P<0{\cdot}05$). (B), Circulatory LOOH levels relative to plasma cholesterol concentration ($\times 10^{-3}$) at bleeding times as indicated. Values are means with their standard errors depicted by vertical bars (*n* 8). Mean values were significantly different between treatments with Lev and the carrier: ANOVA, $P=0{\cdot}002$; Newman–Keuls procedure, $P<0{\cdot}05$. (C), Plot of heart rate (HR) values from (A) *v.* corresponding LOOH values from (B) with r^2 0·79 and $P=0{\cdot}04$ for the slope of the regression function.

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data. Therefore, the fluorescence intensity measured by flow cytometric channel (as shown in Fig. 4 (A)) parallels the cellular content of immunoreactive protein[31]. The results observed by an energetic intake *ad libitum* were expressed relative to those of a restricted intake (Fig. 4 (B)). The results indicated that the intake of ME *ad libitum* increased abundance of cytochrome oxidase, a marker of mitochondrial biogenesis[32], and of eNOS involved in regulating the respiratory rate[33]. Abundance of superoxide dismutase and nitrotyrosine remained unchanged (Fig. 4 (B)).

Impact of lipid-based diet on lipohydroperoxides (Expt 2)

To put to proof a correlation between changes in providing oxidative substrate and LOOH level, the response of circulatory LOOH concentration relative to total cholesterol, LDL cholesterol and HDL cholesterol was measured for starch *versus* lipid (long-chain fatty acids)-enriched diet under isoenergetic and isonitrogenic conditions. Vessel-specific responses of the plasma LOOH concentration (Fig. 5 (A)) and LOOH/total cholesterol ratio were observed (Fig. 5 (B)). Moderate increment (about 20 %) was found in mesenteric arterial plasma when starch energy was replaced by fatty acid energy. In the vein of the mammary gland (peripheral with regard to mesenteric and portal circulation), a lipid-enriched diet decreased the plasma LOOH/cholesterol ratio 1.4 times the ratio observed by feeding a starch-enriched diet (Fig. 5 (B)). Altered results were obtained when plasma LOOH concentration was expressed relative to the concentration of lipoprotein cholesterol. In mesenteric artery plasma, the LOOH/LDL cholesterol ratio was strongly raised (780 %) by replacing carbohydrate energy by lipid energy (Fig. 5 (C)). In portal vein plasma, a corresponding rise in LOOH/HDL cholesterol ratio was found (Fig. 5 (D)) while the LOOH/LDL cholesterol ratio significantly decreased in plasma from the large mammary gland vein (Fig. 5 (C)).

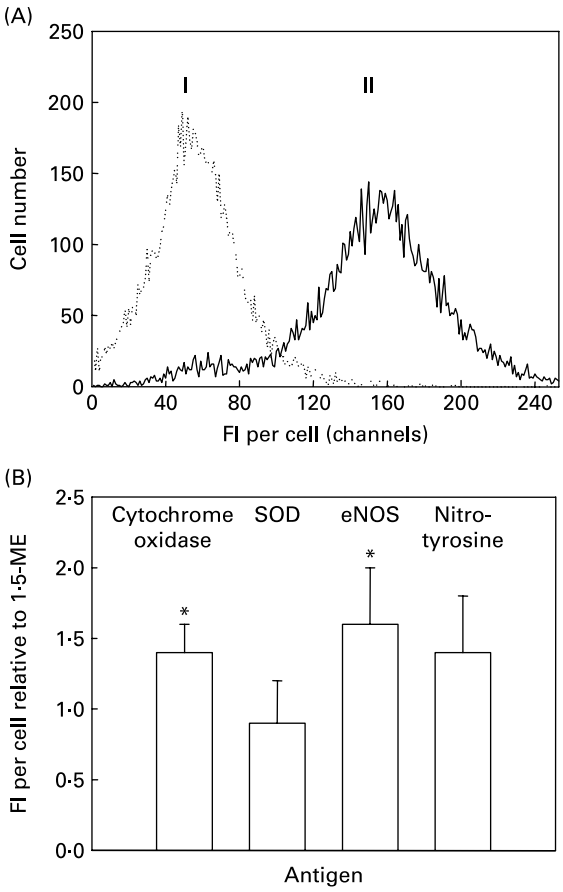

Fig. 4. Dietary effects on the relative abundance of cytochrome oxidase, Cu/Zn superoxide dismutase (SOD), nitric oxide synthase (eNOS) and 3-nitrotyrosine in cardiomyocytes. (A), Representative flow cytometric result obtained by plotting the number of fluorescence-positive cells with subunit IV of cytochrome oxidase as the antigen against fluorescence intensity (FI) per cell (flow cytometric channel number), gating β-actin-positive cells (cardiomyocytes). A shift of FI toward higher values parallels increase in immunoreactive protein per cell. Single cells were prepared by collagenase digestion of heart ventricle specimens. Peak I represents a control, replacing specific antibodies by unspecific ones; peak II denotes incubation with specific Ig followed by fluorescent antispecies Ig in both reactions. (B), Values are means, with their standard errors depicted by vertical bars (*n* 8), expressed by the ratio between *ad libitum* and restricted metabolizable energy (ME) intake. Mean values show significant increase in immunoreactive protein with *ad libitum* ME intake (Dunnett's procedure): $^{*}P < 0{\cdot}05$.

Discussion

Intensity of oxidative metabolism may relate to production of reactive oxygen species exceeding the capacity of the defence system and, as a corollary to this, an increase in LOOH. To investigate whether such a relationship is detectable, the oxidative metabolic rate of the whole body was non-invasively changed by feeding a diet that allows intake of ME *ad libitum* and a diet with moderate-restricted intake. An intake *ad libitum* associated with significantly higher V_{O2} and RER during the periods before and after morning intake subsequently to overnight fasting. The present results are consistent with previous studies reporting meal-induced thermogenesis in ruminants and other species[20,34–35]. We found corresponding changes in the plasma LOOH concentration, utilizing the instability of LOOH to demonstrate transient responses. The LOOH levels were expressed in absolute terms or relative to plasma lipid levels, since human metabolism has been reported to respond to increasing intake of fat with higher plasma lipid concentrations that correlate to a rise in markers of oxidative stress[36]. However, a ME *ad libitum* intake did not substantially change plasma concentrations of lipids probably because the young bulls assimilated the ME provided by the high feeding level for rapid growth (they doubled their daily gain when fed *ad libitum*).

Concentrations of LOOH fluctuated obviously due to formation during the periods of intake and clearance during the intermediary periods. Clearance can be attributed to spontaneous, antioxidant or catalyst-mediated reduction of LOOH to hydroxylated lipids[14], thereby reflecting in part the capacity of the defence against excessive reactive oxygen metabolites. The transient responses differed among diets and the time of intake. The most striking findings were that decreases in absolute LOOH levels or LOOH ratios subsequently to overnight fasting in a ME *ad libitum* intake were lower than in a ME restricted intake, yielding higher circulating LOOH in a ME *ad libitum* intake.

During the intermediate period (after a morning intake and before an intake in the afternoon), clearance of LOOH reduced

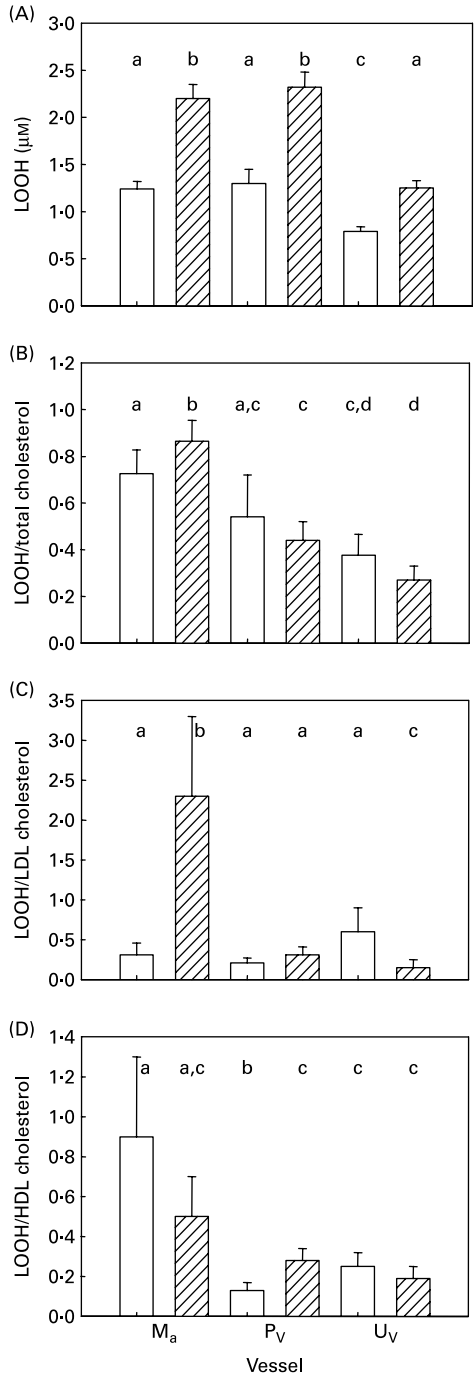

Fig. 5. Diet-induced vessel-specific changes in lipohydroperoxide (LOOH) level and the ratios between LOOH and compartmentalized cholesterol. Plasma from mesenteric artery (M_a), portal (P_v) and a large udder vein (U_v) was obtained following feeding isoenergetic and isonitrogenic diets with starch (□) or protected palmitic and oleic acids (▨) as the major energy source. LOOH were determined in organic phase plasma extracts as described in the Experimental methods section. For clarity, (B), (C) and (D) denote ratios between LOOH and cholesterol concentration $\times 10^{-3}$. Values are means with standard deviations depicted by vertical bars (M_a and P_v, n 12; U_v, n 8). ^{a,b,c} Mean values with unlike superscript letters were significantly different (ANOVA, (A) $P=0{\cdot}0004$, (B) $P=0{\cdot}001$, (C) $P=0{\cdot}009$, (D) $P=0{\cdot}01$; Dunnett's or Dunn's procedure, $P<0{\cdot}05$).

the dietary difference. The responses were similar when expressed in absolute LOOH concentrations or as LOOH/TAG, LOOH/total cholesterol and LOOH/lipoprotein cholesterol ratios owing to small changes in plasma lipids. The similarity of the lipid concentrations is not surprising because a major portion (80–90 %) of the bovine total cholesterol and phospholipids has been reported to be carried by HDL. The present results approximate to the literature data[37–41] as roughly 70 % of the total cholesterol was found in HDL. In addition, feeding level is known[42] to generally alter the amount but not the proportions of digestive end-products. Further, supply of blood metabolites increases with ME intake mainly as a result of a rise in the blood flow to the liver and the portal-drained viscera[42]. This process correlates to heat increment of these tissues and redistribution of the blood seems to relate to vasoactive regulation, including the observed changes in HR.

Of interest is that a ME *ad libitum* intake in the afternoon raised LOOH more strongly than a restricted ME intake. This response can be explained without constraints giving consideration to the temporal course of V_{O2} and RER that informs about preferred oxidation of substrate.

Increasing the plan of bovine feeding generally corresponds to a switch from a low-concentrate diet to a high-concentrate diet[42]. Therefore, ME intake *ad libitum* provided both carbohydrate and lipids, yielding higher basal consumption of O_2 than restricted ME intake. Basal LOOH following overnight fast remained high because lipids are the preferred oxidative substrate when carbohydrates have been consumed by the growing animals. A morning ME *ad libitum* intake recruites carbohydrates and lipids, but carbohydrates are the preferred oxidative substrate as shown by the rise in RER. Therefore, carbohydrate oxidation associates with a lower degree of increase in absolute or relative LOOH than restricted ME intake. The latter is accompanied by a smaller increase in RER, indicative of oxidizing both carbohydrates and lipids with the result of a stronger rise in LOOH than ME *ad libitum* intake. Rapid clearance of LOOH was evident after a ME *ad libitum* intake in the morning. The LOOH levels became similar. The present result resembles the effect of short-term energetic restriction in rats, showing unchanged levels of some oxidative stress markers[18,19].

In the afternoon the significant decrease in RER in response to an *ad libitum* intake indicated preferred oxidation of lipids. The decline in RER associated with a considerable rise in the LOOH concentration. It attained a level observed after morning feeding. A restricted ME intake caused neither decrease in RER nor a striking rise in circulatory LOOH. Thus, oxidative substrate and feeding level are important for generation and clearance of circulatory LOOH.

In turn, energetic restriction causes numerous physiological changes. Therefore, we used an approach independent of feeding to provide support for the correlated responses elicited by dietary intake. Treatment of animals fed ME *ad libitum* with a drug reported to act as an opener for ATP $K_{ATP/ADP}$, an action useful in the therapy of type 2 diabetes[23,30], evoked immediately a transient increase in HR. The rise in HR, which is known as an index of oxidative metabolic rate[20,35], resembled the feeding effect. However, inhibition of HR was expected due to opening cardiac $K_{ATP/ADP}$, which hyperpolarize cardiomyocytes followed by a decrease in cytosolic calcium and concomitantly in the frequency of cardiac

contractions[36,37]. Feeding is known to redistribute the bloodstream toward the digestive tract[42], explaining the observed increase in HR. The instant rise in HR followed by a fall in HR through levcromakalim injected into the jugularis vein is likely to be mediated through an effect on the central nervous system[29,30,43] with HR-regulating $K_{ATP/ADP}$-bearing neurons as targets.

We found (as a novel result to the best of our knowledge) that plasma LOOH relative to plasma cholsterol increased immediately post-levcromakalim whereas the values post-glibenclamide/post-levcromakalim did not significantly differ from control values obtained by treatment with the carrier of the drugs. Therefore, the levcromakalim-induced changes in HR (measured in periods) could be plotted *v.* LOOH (measured at time-points of sampling plasma specimens corresponding to the periods) to demonstrate the approximately linear relationship between HR and plasma LOOH (afternoon post-intake value pair was lacking since blood was not drawn for animal care reasons).

Increase in HR by exercise has been reported to show excessive production of reactive oxygen metabolites, accompanied by oxidatively damaged heart muscle mitochondria[44]. The present results indicate that the concentration of nitrotyrosine, a marker of oxidative damage, did not significantly increase in cardiomyocytes from animals with transiently higher HR and plasma LOOH in response to the energy-dense diet. In turn, this diet increased cardiac immunoreactive cytochrome oxidase to a level similar to the concentration of nitrotyrosine. The present observations seem to be consistent with a higher expression of eNOS protein while the expression of Cu/Zn superoxide dismutase protein was unchanged. Expression of cytochrome oxidase and eNOS has been recently reported to indicate mitochondrial biogenesis with a role of NO in both expression[32,33] and activity of the respiratory chain[45]. Thus, higher immunoreactive cytochrome oxidase and eNOS may indicate mitochondrial biogenesis without impairment of heart cells by oxidative stress. Additionally, NO and eNOS have been implicated in protection against oxidative stress when studying endothelial and smooth muscle cell lines[46,47]. The activity of eNOS is involved in the regulation of vascular relaxation, essential for enhancing the circulatory activity in feeding ME *ad libitum* relative to energetic restriction. In the light of the present results, future study on diet-inducible responses should include vessel-specific analysis.

To obtain evidence for utilization of exogenous lipids (especially long-chain fatty acids) as substrate, the concentration of plasma LOOH was measured in response to a change in the dietary composition. The present results show that replacement of carbohydrate energy by energy provided by long-chain fatty acids induces an increase in absolute plasma LOOH level in vessels supplying intestinum with blood and draining the digestive tract. Lower concentration and response to diets were found in a vessel peripheral to the cardiovascular system (mammary gland vein). Expression of LOOH concentrations relative to compartmentalized cholesterol levels resulted in striking differences between diets and vessel. Because of the anatomical position of portal-drained viscera metabolism of these tissues is sustained by the supply of both end-products of digestion and arterial-blood metabolites. These metabolites (such as acetate, glucose, amino acids), providing the endogenous energy supply, account for about 85 % of the total energy supply[42]. In turn, the ruminant intestine is the major site of *de novo* cholesterol synthesis[41]. Replacement of carbohydrate energy by fat energy has been reported to stimulate intestinal lipoprotein cholesterol export[44] (reflected by portal vein cholesterol). Further, peripheral cholesterol trafficking between lipoproteins is well known[37–40]. Thus, we observed that supplemental fatty acid raised mesenteric artery LOOH moderately relative to total cholesterol while a dramatic rise was observed in LOOH relative to LDL cholesterol. Supplemental fat elevated the level of total cholesterol and HDL cholesterol in portal vein plasma similar to literature data from jugularis or coccygeal plasma[37,40,41]. The present results indicate that the concentration of plasma LOOH depends on intake of dietary lipids in a vessel-specific fashion. We are not aware of any report demonstrating such data. The present results confirm those observed by whole-body gas exchange measurement. Moreover, the responses observed in mesenteric-arterial compared with portal plasma indicate that intestinal activity *per se* did little contribute to peroxidation of lipids. The present results suggest that the vascular endothelium reported to metabolize long-chain fatty acids and to internalize, oxidize and secrete oxidized LDL[3] may be an important source of plasma LOOH.

It should be mentioned that LOOH was measured by a technique capable of measuring oleic acid hydroperoxides[14], since plasma LOOH concentrations were directly determined *via* a hydroperoxide-mediated oxidation of Fe^{2+} to Fe^{3+} and the colour formed by ammonium thiocyanate in an organic phase[14]. Preparation of samples for analysis by more sophisticated methods seems to cause problems in the determination of peroxidized lipids due to moderate recoveries of 25–30 %[48] while recovery of 13-hydroperoxyoctadienoic acid added to plasma averaged 50 (SEM 10) % in the assay used here. Despite this, we measured relative values in response to treatments rather than absolute concentrations. However, in replacing carbohydrate energy by the energy of fat, oleic and linoleic acids accounted for 40 and 9·5 %. Oleic acid is the major unsaturated fatty acid in animal tissues. Its content in lipids can typically be one order of magnitude larger than that of linoleic acid, and two orders of magnitude larger than linolenic acid[14]. Taking into account the relative rates of auto-oxidation (oleic–linoleic–linolenic, 1:30:72), the final relative amounts of LOOH originating from these fatty acids would be comparable[14]. Bovine plasma LOOH concentrations are yet largely unknown, however, and bovine plasma LOOH at restricted intake level agreed with values observed in human plasma using more sophisticated methods[14,49], indicative of reliable results.

In summary, the present data demonstrate production of plasma LOOH in response to feeding and this production is modified by both the time and the level of intake of ME and by the dietary composition. Evidence is provided for a role of the circulatory activity in these transient responses. However, the heart does not appear to be the target of oxidative stress in the bovine model used while vessels seem to be the source of oxidant capable of producing LOOH. Given HR and whole-body gas exchange measures provide information on oxidative metabolic rate, plasma LOOH can associate with this rate in response to feeding after overnight fast. Exogenous long-chain fatty acids appear to be preferred

substrates for lipoperoxidations, suggesting dietary composition may be important for therapeutic interventions in diseases connected with excessive production of reactive oxygen metabolites.

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