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Mechanism of fatty acids induced suppression of cardiovascular reflexes in Rats

Hossam A. Shaltout and Abdel A. Abdel-Rahman

Department of Pharmacology & Toxicology, The Brody School of Medicine at East Carolina University, Greenville, NC 27858.

a) Elevated NEFAs and cardiac baroreflex

b) Dr. Abdel A. Abdel-Rahman

Department of Pharmacology

The Brody School of Medicine

East Carolina University

Greenville, NC 27858

Tel: (252) 744-3470

Fax: (252) 744-3203

abdelrahmana@mail.ecu.edu

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d) Nonstandard Abbreviations:

BRS, baroreflex sensitivity; HR, heart rate; Seq, Sequence; LFα, Low frequency alpha index; HFα, high frequency alpha index; LF_{RRI}, power of beat to beat interval spectrum in the low frequency range; HF_{RRI}, power of beat to beat interval spectrum in the high frequency range; SDRR, standard deviation of beat-to-beat interval; rMSSD, root mean square of successive differences; BPV, blood pressure variability; NEFAs, nonesterified free fatty acids; M2-mAChRs, myocardial M2 muscarinic receptors;

e) Cardiovascular

Abstract:

A blunted baroreflex sensitivity (BRS), impaired heart rate variability (HRV) and high plasma non-esterified fatty acids (NEFAs) are predictors of adverse cardiovascular outcomes. We tested the hypothesis that elevation of NEFAs negatively impacts the cardiac baroreflex response and undertook spectral analyses and molecular studies to delineate the mechanism of action. We employed two interventions to elevate serum NEFAs: (1) overnight fasting (n=7) and (2) i.v. infusion of 1.2 ml/Kg intralipid 20% + heparin (I/H) over 10 min (n=9) in conscious unrestrained male rats. Elevated NEFAs caused by fasting complemented by I/H infusion was associated with a concentration-dependent reduction in spontaneous BRS measured by spectral analysis (LFα, HFα indices) and sequence method (seq-BRS) and HRV measured by frequency domain as power of RRI spectra (LF_{RRI} and HF_{RRI}) and by time domain as standard deviation of beat-to-beat interval (SDRR) and root mean square of successive differences (rMSSD) along with increase in blood pressure variability (BPV) measured as standard deviation of mean arterial pressure (SD-MAP) and power of systolic arterial pressure spectra (LF_{SAP}). Because elevated NEFAs suppressed the vagal component of the baroreflex response (HFα), we tested the hypothesis that NEFA-evoked sequestration of myocardial muscarinic receptor (M2-mAChRs) contribute to the reduced BRS. High NEFAs level was accompanied by increased caveolar sequestration of cardiac M2-mAChRs without changing M2-mAChRs protein expression. We report the first detailed analyses of NEFAs effect on the cardiac baroreflex and show that increased caveolar sequestration of cardiac M2-mAChRs constitutes a cellular mechanism for elevated NEFAs related deleterious cardiovascular outcomes.

Introduction:

Sympathetic over activity and depression of cardiac vagal function contribute to baroreflex dysfunction and may predispose to hypertension (Ketch et al., 2002) and these neural and hemodynamic abnormalities are associated with life threatening cardiac arrhythmias and sudden death (Hennersdorf and Strauer, 2001). Fluctuations in blood pressure and heart rate reflect the dynamic interplay of diverse physiological processes (Akselrod et al., 1985) and are acceptable measures for cardiovascular autonomic balance. Increased heart rate and blood pressure variability and reduced heart rate variability are predictors for mortality (Stein et al., 1994; Lombardi et al., 1996; Task, 1996; Palatini and Julius, 1997; Malliani and Montano, 2002). Spectral analysis of arterial blood pressure and heart rate has provided new insight into the autonomic vascular and cardiac regulation (Cerutti et al., 1994). Analysis of BP and HR variability by the spectral approach, as well as by time domain techniques, represent a useful tool for the study of the mechanisms involved in cardiovascular regulation in both normal and diseased conditions (Parati et al., 1995b).

Elevated plasma lipids have been linked to many cardiovascular diseases and are associated with an increase in sudden death (Jouven et al., 2001; Wyne, 2003). Patients with familial combined hyperlipidemia have elevated plasma NEFAs (Carlsson et al., 2000) and higher BP (Castro Cabezas et al., 1993). Moreover, plasma NEFAs measured after an overnight fast and 2 h after an oral glucose load independently predicted the development of hypertension (Fagot-Campagna et al., 1998). It is imperative to note that short term elevation in plasma NEFA following the acute infusion of intralipid in humans, caused impairment of baroreflex measured by the Oxford (phenylephrine)

method (Gadegbeku et al., 2002). A comprehensive investigation of the impact of elevated plasma NEFAs on autonomic control of cardiac baroreflex and indices has not been reported.

At the cellular level, high fat diet decreases M2 muscarinic cholinergic receptors (M2-mAChRs) number and function in cardiomyocytes (Pelat et al., 1999). Notably, caveolar sequestration plays a role in M2-mAChRs desensitization (Feron et al., 1997; Dessy et al., 2000). The possibility has not been investigated that short-term elevation in plasma NEFA may cause sequestration of myocardial M2-mAChR, which might explain the adverse effect of NEFA on the vagal component of the baroreflex arc. Undoubtedly, the elucidation of the temporal correlation between elevation of NEFAs levels and its hemodynamic effects and providing a possible mechanism for these hemodynamic changes are clinically important.

In this study we investigated the effect of short term (15 hr) elevation of NEFAs levels in conscious freely moving rats by: (i) overnight fasting, and (ii) overnight fasting + intralipid/heparin infusion on the systolic arterial pressure (SAP), heart rate (HR), spontaneous baroreflex sensitivity (BRS) measured by RRI and systolic arterial pressure (SAP) cross spectral analysis (low frequency alpha index; LFα and high frequency alpha index; HFα). Also we investigated the effect of NEFAs elevation on spontaneous baroreflex measured by the time domain analysis (sequence method). We followed the effect of NEFAs elevation on HRV measured by frequency domain analysis as $LF_{RRI\ and}$ HF_{RRI} and time domain analysis as standard deviation of beat-to-beat interval (SDRR) and root mean square of successive differences (rMSSD). Because the spectral analyses findings provided evidence that elevated plasma NEFA was associated with suppressed myocardial vagal tone, we

hypothesized that caveolar sequestration of M2-mAChRs in cardiac myocytes may underlie the NEFAs induced attenuation of the activity of the parasympathetic limb of baroreflex arc and the associated increase in heart rate and attenuation of HRV in fasting and intralipid/heparin treated conscious freely moving rats. These studies were undertaken in conscious rats to circumvent the confounding effects of anesthesia on data interpretation. Notably, the rat is considered an appropriate model for generating clinically relevant data on lipid regulation and cardiovascular responses to clinically prescribed medications where fatty acid oxidation and metabolism in rats and humans are similar (Stanley et al., 1997).

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Materials and Methods

Preparation of the rats: Male Sprague-Dawley rats (300-350 g; Harlan Farms, Indianapolis, IN) were housed in a room with controlled environment at constant temperature of 23±1°C, humidity of 50±10% and were maintained on a 12-12 h light-dark cycle with light off at 19:00 h. The rats had free access to water and food. Arterial blood pressure was measured according to the method used in our previous studies (Shaltout and Abdel-Rahman, 2003). Briefly, the rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Catheters (polyethylene 10 connected to polyethylene 50), filled with heparinized saline (100 U/ml), were placed in the abdominal aorta and vena cava via the femoral artery and vein for measurement of BP and i.v. administration of drugs, respectively. The catheters were tunneled s.c., exteriorized at the back of the neck between the scapulae, and plugged by stainless steel pins. Incisions were closed by surgical staples and swabbed with povidone-iodine solution. Each rat received buprenorphine hydrochloride (Buprenex; 30 μg/kg; s.c) to control pain and 50,000 units/kg of benzathine and penicillin G procaine in an aqueous suspension (Durapen; i.p) and was housed in a separate cage. Each experiment started 2 days after surgery, by connecting the arterial catheter to a Gould-Statham pressure transducer (Oxnard, CA). Blood pressure and heart rate were recorded and analyzed using Gamma 4 data acquisition and analysis system (Grass Instrument Division, Astro-Med, Inc. West Warwick, RI). BP and HR were also displayed on a Grass polygraph (model 7D, Grass Instruments Co., Quincy, MA). The venous catheters were used to infuse I/H mixture or saline. On the experiment day blood samples were collected for the measurement of plasma NEFAs concentration by means of an enzymatic colorimetric method using a commercial kit (NEFA C, wako chemicals, GmbH)

Experiments were performed in strict accordance with institutional animal care and use guidelines, and in accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Frequency Domain Analysis: Spontaneous baroreflex sensitivity was calculated by the frequency-domain analysis method as in reported studies (Parati et al., 1995a) using a newly developed software designed for rats (Nevrokard SA-BRS**,** Medistar, Ljubljana, Slovenia). **Nevrokard™ SA-BRS** is a software package for baroreflex sensitivity analysis in small animals. It features frequency domain analysis of up to 5 Hz and a re-sampling rate of 10 Hz (http://www.nevrokard.medistar.si/maini/brs.html).

Power spectral densities of systolic arterial pressure (SAP) and RR intervals (RRI's) oscillations were computed by 512 points Fast Fourier Transform (FFT) and integrated over the specified frequency range (LF; 0.25-0.75 Hz) and (HF; 0.75-5.0 Hz). Basically the SAP and RRI files generated via the data acquisition system (Gamma 4, Grass Instrument Division, Astro-Med, Inc., West Warwick, RI) at 1000 HZ were analyzed using Nevrokard SA-BRS software. A Hanning window was applied and the spectra of SAP and RRI series, and their squared-coherence modulus, were computed if the coherence is greater than 0.5 in accordance with reported criteria (Parati et al., 1995a). The square-root of the ratio of RRI's and SAP powers were computed to calculate LF, HF alpha indices, which reflect the baroreflex sensitivity (Parati et al., 1995a).

Power of RRI spectra in LF, HF range (LF_{RRI} and HF_{RRI}) were calculated in normalized unites and the ratio of LF_{RRI}/HF_{RRI} was used as a measure of sympathovagal balance (Laitinen et al., 1999). Power of Systolic arterial pressure spectra was calculated as LF_{SAP} as a measure of blood pressure variability (BPV).

Sequence Method: Baroreflex sensitivity calculated by this method is based on quantification of sequences of at least three beats (n) in which systolic arterial pressure (SAP) consecutively increases (UP sequence) or decreases (DOWN sequence), which are accompanied by changes in the same direction of the RR intervals (RRI's) of the subsequent beats (n+1). In order to be included in the BRS estimate, each sequence must fulfill the following criteria (Wang et al., 2004): (1) minimal RRI change 3 ms; (2) minimal SAP change 1 mmHg; (3) minimal number of beats, 3 or more, in the sequence; (4) minimal correlation coefficient of 0.85. The software scans the RRI and SAP records, identifies sequences, and then calculates linear correlation between RRI and SAP for each sequence. If the correlation coefficient exceeds a pre-set critical value (0.85), the regression coefficient (slope) is calculated and accepted. The mean of all individual regression coefficients (slopes), which is a measure of sequence BRS was then calculated. Overall, three parameters were obtained by this method (Sequence BRS- SAP UP, DOWN and TOTAL).

Time-Domain Analysis: Three time-domain parameters were used to measure hemodynamic variability as in previous studies (Stein et al., 1994; Sgoifo et al., 1997). Heart rate variability was determined by computing the standard deviation of beat-to-beat interval (SDRR) and the root mean square of successive beat-to-beat differences in R-R interval duration (rMSSD). The standard deviation of the mean arterial pressure (SDMAP) was used as a measure for blood pressure variability.

Immunoblotting: Ventricular tissue was homogenized in a homogenization buffer {50 mM Tris (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 2 μm leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% (vol/vol) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate}. After

centrifugation (12,000 *g* for 5 min), protein was quantified in the supernatant using a modified Lowry assay procedure (the Bio-Rad Protein assay system). For Western blot analysis of M2 and caveolin-3, 40µg were loaded onto Novex Bis-Tris 4-12% gels and run according to the manufacture manual for best resolution. Proteins were transferred to nitrocellulose membranes after electrophoresis. After being blocked with 5% nonfat dry milk in Tris-buffered saline (TBS), the blots were incubated with the specified primary antibody anti-M2 dilution at 1: 300 (Chemicon International; Temecula, CA) and anti-caveolin-3 dilution at 1:2,500 (Transduction Laboratories; San Diego, CA), in TBS buffer containing 5% nonfat dry milk overnight at 4°C. After four washes, the blots were incubated for 2 hours at room temperature with secondary antibody diluted in TBS buffer containing 5% nonfat dry milk. After three additional washes in TBS buffer with 0.1% (vol/vol) Tween 20, the blots were detected by an enhanced chemiluminescence system (ECL) (Amersham) and exposed to X-ray film at room temperature. Films were developed in KodaK D-19 developer and analyzed on a video based computerized system. Protein levels were quantified by measuring the optical density of the specific bands on Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) (Shaw et al., 1995). The protein concentration was presented as percentage from the control sample loaded in the same gel.

Immunoprecipitation: Ventricular tissue lysates were prepared using the same method described for immunoblotting. Lysates containing 1 mg of detergent soluble protein were precleared by incubating with Protein A-Sepharose for 1 h at 4°C and then transferred to a fresh tube. Five µl of anti-M2 polyclonal antibody (10 µg/ml; Chemicon International) were added and gently mixed overnight at 4°C. Thirty microliters of 50% Protein A-

Sepharose slurry were added to the mixture. After an overnight incubation rotating at 4°C, immune complexes were collected by centrifugation, washed three times with 1 ml of immunoprecipitation buffer and once with wash buffer (50 mM Tris, PH 8). The complexes were disrupted by heating at 95^0C for 3 minutes in 30 µl immunoblotting sample buffer (100 mM Tris, 100 mM DTT, 1% SDS , PH 7.5). The supernatant was then analyzed by Novex Bis-Tris 4-12% gels, followed by protein immunoblotting for caveolin-3 using anti-caveolin-3 dilution of 1:2,500 (Transduction Laboratories; San Diego, CA) followed by analysis as described above.(Dessy et al., 2000).

Protocols and experimental groups:

15 conscious SD male rats were used 48 hr after implantation of femoral catheters. The rats had free access to food and water. Following a stabilization period of at least 30 min after connecting the arterial and venous lines to the pressure transducer and an i.v. delivery system, respectively, an arterial blood sample (0.25 ml) was drawn for the measurement of basal NEFA level. MAP, HR, BRS (as LFα, HFα, seq BRS SAP-UP, seq BRS SAP-DOWN, seq BRS SAP-TOTAL), HRV (LF_{RRI}, HF_{RRI}, SDDN and rMSSD), LF_{RRI}/HF_{RRI} ratio and BPV as LF_{SAP} and SDMAP were measured over a period of 2 hours as detailed earlier. The rats were then subjected to overnight fasting (water ad lib) and randomly divided into 2 groups: (i) control (n=6) and (ii) intralipid/heparin (I/H) treated (n=9). I/H was infused as a 20% intralipid/heparin mixture (Sigma Chemicals) at dose 1.2 ml/kg over 10 min; (Widmaier et al., 1992; Fabris et al., 2001; Gadegbeku et al., 2002) , same volume of heparinized saline was infused in the control group. Plasma NEFA and the hemodynamic parameters were measured at 30, 60, 180 min after I/H or saline infusion, which was followed by euthanasia and tissue collection.

In a separate group of 7 conscious male Sprague-Dawley rats, catheterized as detailed above for BP and HR measurement, BRS was measured by the Oxford (phenylephrine) method daily for 5 days as in our previous studies (El-Mas and Abdel-Rahman, 1997; Shaltout and Abdel-Rahman, 2003). Briefly 4-5 randomized bolus doses of phenylephrine (1-16 μg/kg) were injected at 5-min intervals. In each rat, the peak changes in MAP and HR, obtained following phenylephrine injections, were used for the construction of the baroreflex curve and the slope of the regression line represented the BRS. In the same rats, BRS was measured by Nevrokard SA-BRS software as detailed above.

Drugs. Phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO), Buprenex (Buprenorphine hydrochloride; Rickitt & Colman, Richmond, VA), Durapen (penicillin G benzathine and penicillin G procaine; Vedco, Overland Park, KS), heparin (Elkins-Sinn Inc., Cherry Hill, NJ), Intralipid 20% (Phospholipid stabilized soybean oil, which contains Soybean Oil 20.0g, Phospholipids 1.2g, Glycerin, USP 2.25g, Sigma-Aldrich, st. Louis, MO), pentobarbital sodium (Sigma-Aldrich, st. Louis, MO), sterile saline (B. Braun Medical Inc., Irvine, CA) and NuPAGE Novex Bis-Tris Gels (Invitrogen, Carlsbad, CA) were purchased from commercial vendors.

Statistical Analysis

Values are expressed as means ± SEM. GraphPad (San Diego, CA) Instat statistical analysis software (ANOVA with repeated measures followed by Bonferroni post hoc analysis) was used for multiple comparisons. Probability levels less than .05 was considered significant.

Results:

Baseline Data

Baseline values of NEFAs plasma level, baroreflex sensitivity, blood pressure, heart rate, and their variability indices measured in ad lib fed freely moving conscious male rats, which subsequently received the treatment (I/H) or served as control were not statistically different.

There was no change in plasma NEFAs levels, BRS, BP, HRV and BPV in ad-lib group that received the same volume of heparinized saline (data not shown).

Effect of Fasting and Intralipid/Heparin on Plasma NEFAs Level:

As shown in Fig. 1C, plasma NEFAs was elevated by overnight fasting by approximately 40%. Plasma NEFA remained unchanged in the control group (overnight fasting) throughout the 3 hr observation period. However, in the treatment group, a 10 min I/H infusion caused substantial increase in plasma NEFAs, which peaked at 30 min and tended to decline to pre-infusion level by 3 hr (Fig. 1C).

Effect of Fasting and Intralipid/Heparin on Blood Pressure and Heart Period:

As shown in Fig. 1A, overnight fasting caused a slight increase in SAP. I/H infusion caused statistically significant increase in SAP, which started during the infusion, peaked (13%) at 1 hr and remained significantly higher than the corresponding control value at 3 hr. Beat to beat interval (RRI) was slightly but significantly reduced by overnight fasting (~4%) and I/H infusion caused a further reduction, which reached its nadir at 30 min and remained significantly lower than the control values over the 3 hr observation period (Fig. 1B).

Effect of Fasting and Intralipid/Heparin on Baroreflex Sensitivity:

Overnight fasting significantly reduced BRS measured by the spectral analysis (Fig. 2, A and B) and sequence method (Fig. 2, C, D and E) in rats that subsequently received I/H infusion or heparinized saline (control). LF-α (Fig. 2A) and HF-α (Fig. 2B) were reduced 25-30% following overnight fast. I/H infusion caused a statistically significant reduction in total BRS, which reached its nadir at 30 min and tended to recover but was still significantly lower than the control value at 3 hr (Fig. 2, A, B and E). The reduction caused by I/H in BRS was more evident with the HF-α (Fig. 2B) than the LF- α (Fig. 2A) component (spectral method) and SAP-UP (Fig. 2C) than SAP-DOWN (Fig. 2D) (sequence method).

Effect of Fasting and Intralipid/Heparin on Heart Rate and Blood Pressure Variability:

Overnight fasting significantly reduced heart rate variability measured by spectral analysis as HF_{RRI} (Fig, 3B) and by the time domain method as SDRR and rMSSD (Fig. 3, C& D) in rats that subsequently received I/H infusion or heparinized saline (control). HF_{RRI} (Fig, 3B), SDRR and rMSSD (Fig. 3, C& D) were reduced significantly following overnight fasting. I/H infusion caused a statistically significant reduction in HRV, which reached its nadir at 30 min and tended to recover but was still significantly lower than the control value at 3 hr (Fig. 3, B, D). Meanwhile, overnight fasting increased LF_{RRI} and the sympathovagal balance index LF_{RRI}/HF_{RRI} (Fig, 3 A,E) and blood pressure variability measured by spectral analysis as LF_{SAP} (Fig. 4A) and time domain analysis as SDMAP (Fig. 4B). LF_{RRI} and LF_{RRI}/HF_{RRI} ratio was significantly increased by overnight fasting (80%, 115% respectively). I/H infusion caused further increase (170 %, 300 %) by 30 min and both variables were still significantly higher than control value at 3 hr. LF_{SAP} and

SDMAP (Fig. 4A, B) were significantly increased by overnight fasting (approx. 25%) and was further increased by I/H infusion (approx. 50%) at 30 min and remained significantly higher than control till the end of recording period (Fig. 4A, B).

Effect of Fasting and Intralipid/Heparin on Cholinergic Muscarinic Receptor (M2) and Caveolin-3 (C3) Protein Expression and their Association in Rat Cardiac Myocytes:

M2 expression in overnight fasting and I/H treated overnight fasting rats was not significantly different from ad-lib rats (Fig. 5A). Overnight fasting alone and in combination with intralipid/heparin infusion significantly increased C3 levels compared to ad-lib control. (Fig. 5B**)**. The caveolar sequestration of M2 mAChRs (association between M2 & C3 measured by immunoprecipitation) was significantly increased, from ad-lib control, by overnight fasting (40%). I/H infusion following overnight fasting further increased the association between M2 & C3 (66 %) (Fig. 5C).

Correlation between Baroreflex Sensitivity and Nonesterified Fatty Acids

Baroreflex sensitivity, measured as HFα, was highly correlated with plasma NEFAs levels (Fig. 6A). The data included in Fig. 6A were collected from rats when they were fed ad lib (before any treatment) and following overnight fasting in both treated and control groups. The data show a highly significant inverse relationship between BRS and NEFAs.

Correlation between Baroreflex Sensitivity measured by Oxford Method and by Spectral Analysis

As shown in Fig. 6B, the baroreflex sensitivity measured by the Oxford method and by Nevrokard- SA-BRS software in conscious freely moving rats were highly correlated ($r = 0.98$; $p<0.05$).

Discussion:

We investigated the effect of short-term elevation of plasma NEFAs using two interventions: i) overnight fasting and ii) overnight fasting followed by infusion of intralipid/heparin mixture on indices of baroreflex sensitivity and blood pressure, heart rate and their variability. The frequency domain and time domain measurements of blood pressure and heart rate variability were employed to determine changes in cardiovascular autonomic control. We also tested the hypothesis that enhanced caveolar sequestration of myocardial M2 muscarinic cholinergic receptors could provide a cellular mechanism of NEFAs induced attenuation of the vagal component of the cardiac baroreflex response in conscious freely moving SD male rats.

The main findings in this study are (i) intralipid/heparin infusion significantly elevated plasma NEFAs levels, (ii) elevation of plasma NEFAs by overnight fasting followed by intralipid/heparin infusion significantly increased systolic arterial blood pressure and its variability, (iii) intralipid/heparin infusion significantly increased heart rate and reduced its variability, (iv) elevation of NEFAs significantly increased the power of RRI at LF range and reduced it in the HF range causing a significant increase in the LF_{RRI}/HF_{RRI} ratio, a measure for sympathovagal balance, (v) NEFAs elevation significantly increased the association between M2-mAChRs and Caveolin-3 without changing myocardial M2-mAChRs or caveolin-3 protein level.

We show, for the first time in conscious rats, that acute elevation of NEFAs caused baroreflex dysfunction over a period of 3 hr after intralipid infusion, which agrees with the finding that acute elevation of plasma NEFAs reduces baroreflex measured by phenylephrine infusion in humans (Gadegbeku et al., 2002).

We found that elevation of NEFAs with overnight fasting significantly attenuated LFα coefficient and HFα calculated by cross spectral analysis and also attenuated the time domain measures of the baroreflex (Seq BRS- SAP UP, DOWN, TOTAL). We used two non-pharmacological methods for BRS measurement. The reduction in BRS caused by elevated NEFAs measured by the spectral analysis and time domain methods in the present study fully agrees with the clinical findings using the phenylephrine method (Gadegbeku et al., 2002). However, the methods used in the present study offered the opportunity to obtain a detailed analysis of the BRS and other cardiac indices on beat-by-beat basis. Equally important, we circumvented the phenylephrine evoked increase in BP, which if applied could have confounded the data interpretation. The phenylephrine method is based on open-loop model, in which the increases in RR- interval and BP are related according to a linear model, while the methods used in our study provide a closed loop estimation of BRS, in which blood pressure oscillations induce changes of RR- interval that in turn are able to modify blood pressure (Pitzalis et al., 1998). Notably, phenylephrine at 10 μg/kg which falls within the doses used to construct BRS curve by Oxford method in reported studies including ours (Gadegbeku et al., 2002; Shaltout and Abdel-Rahman, 2003) reduced the plasma NEFAs level (Imura et al., 1971). Further, the sequence BRS is an accepted measure for tonic parasympathetic cardiac control (Wang et al., 2004). Undoubtedly, the present findings with two different methods bolster our conclusion about the status of the baroreflex following evoked increases in plasma NEFAs.

Intralipid/heparin infusion in overnight fasting rats caused a further attenuation in HF-α and Seq BRS-SAP (UP, DOWN and TOTAL) that showed a recovery tendency but remained significantly lower (HF-α and Seq BRS- TOTAL) than the control group at the end

of the study (3hr). Two findings suggest a strong link between increased NEFAs and the reduced vagal component of the cardiac baroreflex. First, we demonstrated that the increase in NEFAs preceded the BRS dysfunction. Second, we demonstrated for the first time, a significant inverse correlation between plasma NEFAs and BRS (Fig. 6A). Our conclusion is bolstered by the immunoprecipitation findings and that Seq BRS-SAP UP (index of parasympathetic control) was more affected by overnight fasting than Seq BRS-SAP DOWN, (index of sympathetic control) (Parati et al., 2001).

Because we used new software for the measurement of BRS as LFα and HFα, we undertook a preliminary study to validate the software. In this validation study, we compared the baroreflex sensitivity measured in the same rat by the Oxford (phenylephrine) method (open loop) and by Nevrokard- SA-BRS software (closed loop). The rats used in the validation study and in the present study were of similar age and weight. The data generated showed a highly significant correlation between the BRS values obtained by the two methods (Fig 6B). There are, however, differences between the reported open-loop gain values in rats, which varied between 1.3 to 2.5 ms/mm Hg with most of the studies reporting values of 1-1.5 ms/mm Hg (El-Mas and Abdel-Rahman, 1997; Mosqueda-Garcia et al., 1998; Shaltout and Abdel-Rahman, 2003). The HFα value obtained in the present study (2.5 ms/mmHg) is similar to the higher end of the reported range for the open loop gain.

It is noteworthy that overnight fasting, a standard experimental procedure followed in most of clinical and experimental studies, per se can attenuate baroreflex sensitivity and alter cardiovascular indices. For example, our experimental design resembled the clinical setting where the patients fasted overnight before receiving intralipid/heparin infusion (Gadegbeku et al., 2002). We found that overnight fasting slightly increased SAP, and that further (16

mmHg) increase occurred 60 min following I/H infusion in conscious rats. Our finding that acute elevation of NEFAs increase SAP supports the epidemiological finding that elevated NEFAs predict the development of hypertension (Fagot-Campagna et al., 1998) and that elevation of NEFAs by 3 hours of intralipid infusion increases BP (Manzella et al., 2001). The increase in BP could be explained by the ability of fatty acids to impair endothelial function (Davda et al., 1995) and to enhance vascular α_1-adrenoceptor signaling (Stepniakowski et al., 1996; Haastrup et al., 1998). The elevation in BP after acute elevation in NEFAs could also be due to activation of sympathetic activity inferred by the increased LF_{SAP} and SD-MAP, accepted markers of sympathetic activity (Julien et al., 1995) and baroreflex dysfunction. This increase in sympathetic activity could also be responsible for the baroreflex resetting. It is noteworthy that in the present study overnight fasting (10-12 hours), as in other studies (Halliwell et al., 1996) increased free fatty acids, which may explain the modest increase in mean arterial pressure. Prolonged fasting involves other metabolic changes, which may overwhelm the moderate increases in plasma NEFAs and sympathetic activity and cause reduction in blood pressure.

Elevated heart rate and reduced HRV are independent predictors for cardiovascular and noncardiovascular mortality (Stein et al., 1994; Palatini and Julius, 1997; Palatini, 1999; Seccareccia et al., 2001). We found that overnight fasting and/or I/H infusion increased HR, which could be due to enhancement of the sympathetic tone and/or inhibition of the parasympathetic tone. In dogs, acute elevation of NEFAs causes tachycardia mainly due to impairment of parasympathetic control (Verwaerde et al., 1999). Overnight fasting and I/H infusion increased LF_{RRI} and reduced HF_{RRI} and hence the LF_{RRI}/HF_{RRI} ratio, a measure of sympathovagal balance (Laitinen et al., 1999), which suggests an enhancement of

sympathetic control and a reduction in parasympathetic tone. These findings agree with similar findings in humans obtained following 3-hr intralipid infusion (Manzella et al., 2001). Both SDRR and rMSSD, which primarily reflect the vagal activity of the heart (Stein et al., 1994), were reduced by overnight fasting and further reduced by I/H infusion. This suggests that the shift towards the sympathetic dominance results from the lack of a sufficient parasympathetic counteraction to sympathetic activation (Sgoifo et al., 1999). This also may explain the tachycardia associated with elevation of NEFAs levels. It is noteworthy that elevation of plasma NEFAs by overnight fasting and intralipid infusion increased both blood pressure and heart rate, which suggests possible involvement of the central sympathetic nervous system activation.

We report for the first time that elevation of NEFAs via overnight fasting followed by I/H infusion caused an enhancement of caveolin-3 protein expression and its association with M2-mAChRs without significantly changing M2-mAChRs expression. We reasoned that NEFAs evoked myocardial M2 association with C3 might account, at least in part for the attenuated vagal component of the cardiac baroreflex measured by spectral analysis. Caveolae, are now recognized to be plasma membrane compartments with distinct lipid and protein composition that sequester and regulate the function of cytoplasmically oriented signal transduction molecules (Okamoto et al., 1998). Notably, caveolar sequestration plays a role in desensitization and inactivation of many G protein coupled receptors (GPCRs) including M2-mAChRs receptors (Dessy et al., 2000; Feron and Kelly, 2001). It may be argued that the time after intralipid infusion (minutes to hours) might not be long enough to allow the translocation and association between the M2-mAChRs and C3 and therefore precludes such a cellular mechanism as an explanation for the attenuated cardiac vagal component following plasma

NEFAs elevation. Findings from the present study and reported studies argue against this notion. In the present study, baroreflex dysfunction (attenuated vagal component) following overnight fasting was associated with increased expression of C3 and its association with myocardial M2-mAChRs (Fig. 5 B and C). Translocation of M2-mAChRs and its association with C3 occurs in the myocardium within minutes following the addition of M2-mAChRs agonist (Feron et al., 1997). Caveolar sequestration of GPCRs or enzymes (e.g. eNOS) occurs even in the absence of significant change in the expression of C3 (Dessy et al., 2000; Feron and Kelly, 2001). Together these findings support the view that enhancement of M2-mAChRs C3 association contributes to the NEFAs evoked baroreflex dysfunction.

In summary, the current study sought evidence to characterize the role of caveolar sequestration of cardiac muscarinic receptors (M2-mAChRs) in hemodynamic changes elicited by elevated NEFAs levels as a result of overnight fasting and intralipid/heparin infusion. This study is the first to provide experimental evidence that implicates a cellular mechanism for NEFAs induced attenuation of BRS and HRV parameters and increased heart rate through enhancement of caveolar sequestration of cardiac M2-mAChRs, which results in M2-mAChRs inactivation and attenuation of the parasympathetic control on the heart. The results of this study may explain, at least partly, the mechanism that underlies the adverse cardiovascular outcomes associated with high plasma NEFAs in the general population.

Study limitation. Due to the limited quantities of atrial tissue, we measured caveolin 3 and M2-mAChRs protein levels and their association in ventricular myocytes. We acknowledge that activation by acetylcholine of the atrial M2-mAChRs determines the contribution of the vagal component to heart rate control and to the baroreflex response.

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Footnote

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Legends for Figures

Fig. 1. Effect of overnight fasting (O) and overnight fasting + intralipid/heparin (●) infusion on systolic arterial pressure (SAP; mmHg, **A**), inter beat interval (RRI, ms, **B**) and non-esterified fatty acids (NEFAs, meq/L, **C**) in conscious unrestrained rats. Intralipid/heparin or saline was infused over 10 min. Values are expressed as means ± SE. $ P<0.05 vs. baseline at day 1 (ad-lib), * P<0.05 vs. control (fasting), # P<0.05 vs. I/H baseline at day 2 (0 min).

Fig. 2. Effect of overnight fasting (O) and overnight fasting + intralipid/heparin (●) infusion on baroreflex sensitivity (BRS) measured as (LF-α, **A**), (HF- α, **B**), (seq-BRS SAP-UP, ms/mmHg, **C**), (seq-BRS SAP-DOWN, ms/mmHg, **D**) and (seq-BRS SAP-TOTAL, ms/mmHg, **E**) in conscious unrestrained rats. Intralipid/heparin or saline was infused over 10 min. Values are expressed as means ± SE. $ P<0.05 vs. baseline at day 1 (ad-lib), * P<0.05 vs. control (fasting), # P<0.05 vs. I/H baseline at day 2 (0 min).

Fig. 3. Effect of overnight fasting (O) and overnight fasting + intralipid/heparin (●) infusion on heart rate variability (HRV) measured as power of RRI spectra in LF range (LF_{RRI}, **A**), Power of RRI spectra in HF range (HF_{RRI}, **B**), standard deviation of beat-to-beat interval (SDRR, ms, **C**) and the root mean square of successive beat-to-beat differences in R-R interval duration (rMSSD, ms, **D**) and the sympathovagal balance index (LF_{RRI}/HF_{RRI}, **E**) in conscious unrestrained rats. Intralipid/heparin or saline was infused over 10 min. Values are expressed as means ± SE. $ P<0.05 vs. baseline at day 1 (ad-lib), * P<0.05 vs. control (fasting), # P<0.05 vs. I/H baseline at day 2 (0 min).

Fig. 4. Effect of overnight fasting (O) and overnight fasting + intralipid/heparin (●) infusion on blood pressure variability (BPV) measured as Power of systolic arterial pressure spectra in LF range (LF_{SAP}, **A**) and standard deviation of mean arterial pressure (SD-MAP, mmHg, **E**) in conscious unrestrained rats. Intralipid/heparin or saline was infused over 10 min. Values are expressed as means ± SE. $ P<0.05 vs. baseline at day 1 (ad-lib), * P<0.05 vs. control (fasting), # P<0.05 vs. I/H baseline at day 2 (0 min).

Fig. 5. Effect of overnight fasting (▒) and overnight fasting + intralipid/heparin (▉) infusion on M2 muscarinic cholinergic receptors expression (**A**), Caveolin-3 expression in rat ventricles (B) and M2 caveolin-3 association (**C**) in the ventricles as percentage to the Ad-Lib control group ( ). Intralipid/heparin or saline was infused over 10 min. Tissues are collected at 3 hours after start of infusion. Values are expressed as means ± SE. * P<0.05 vs. Ad-lib control, # P<0.05 vs. fasting control.

Fig. 6. A. Correlation between plasma nonesterified fatty acids (NEFAs) levels and baroreflex sensitivity measured as power of RRI and SAP cross spectral analysis in frequency range 0.75-5.0 Hz (HFα, ms/mmHg) in ad lib and overnight fasting rats **(A;** mEq/L, milliequivalent per liter**)**. **B.** Correlation between baroreflex sensitivity measured daily for 5 days by the Oxford method and by power of RRI and SAP cross-spectral analysis in frequency range 0.75-5.0 Hz (HFα, ms/mmHg).

Fig 1

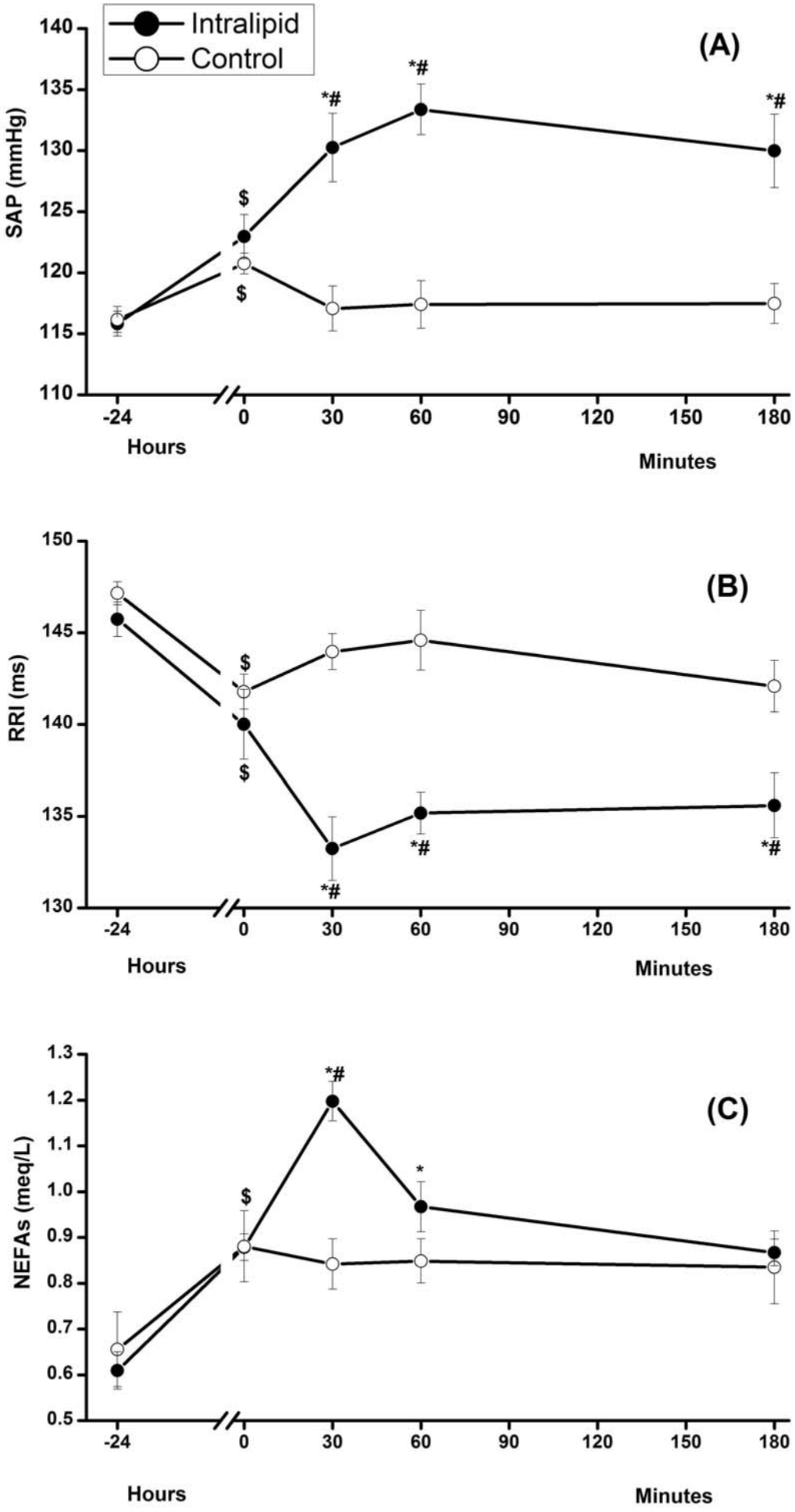

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Fig. 2

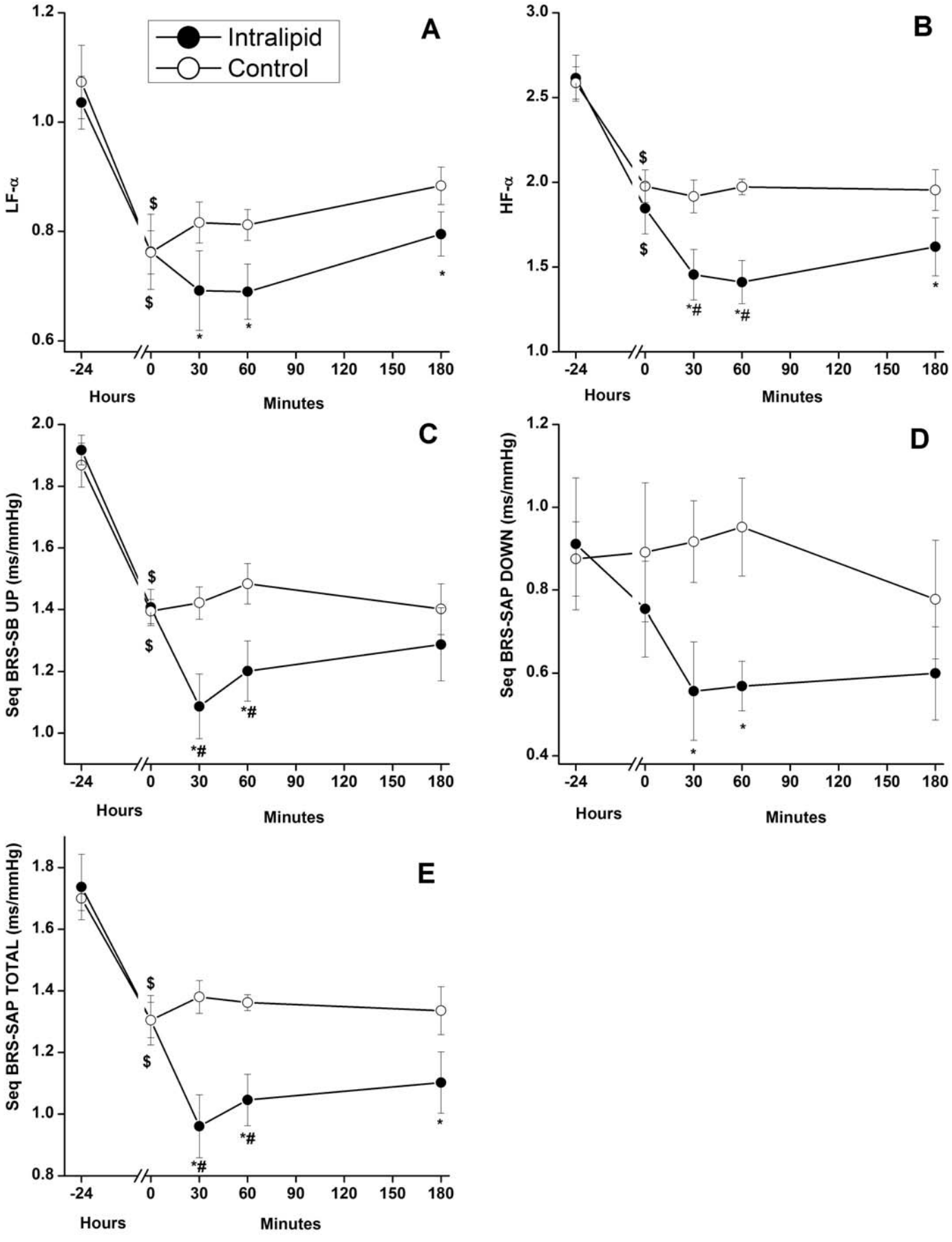

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Fig. 3

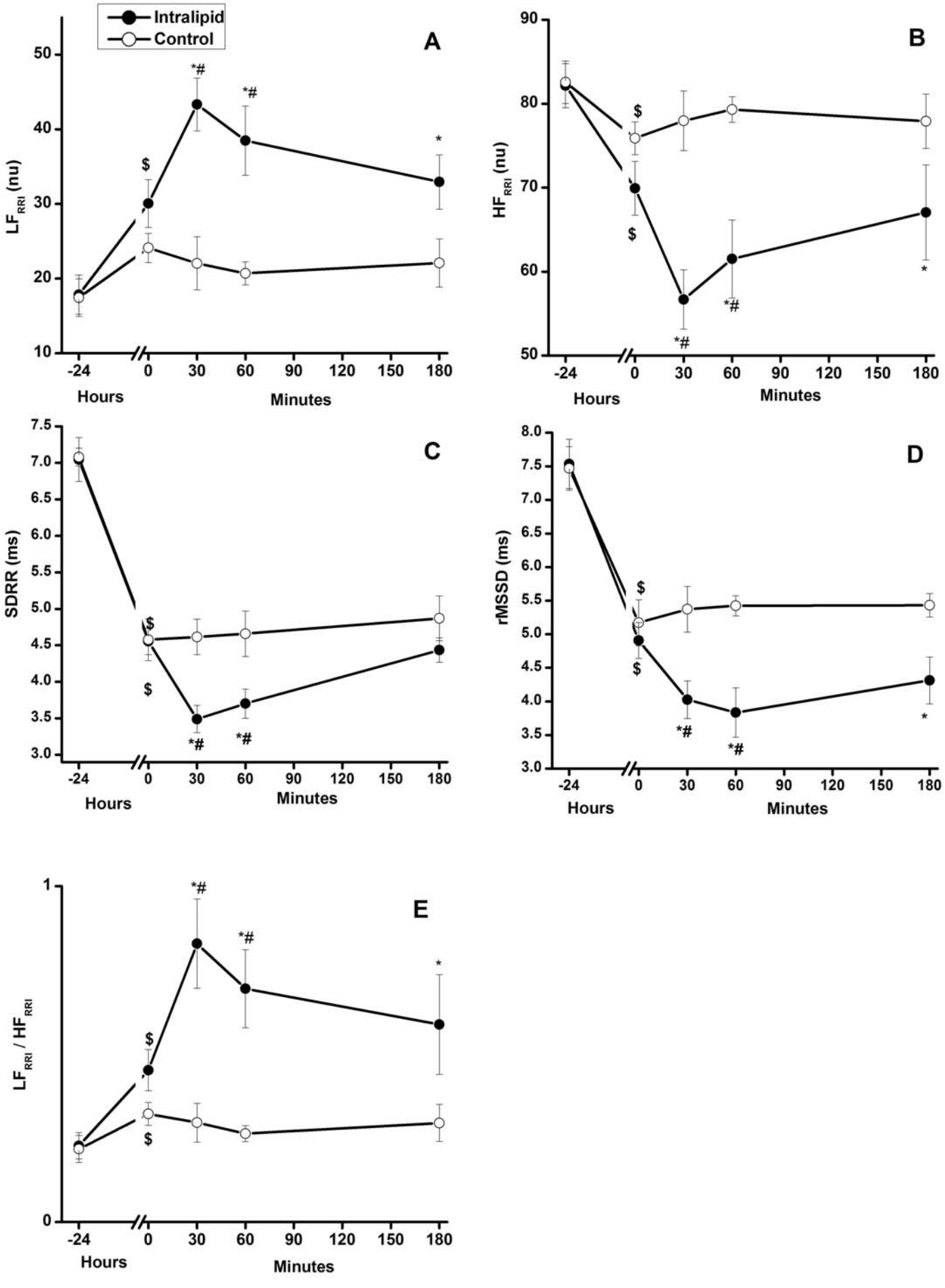

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Fig. 4

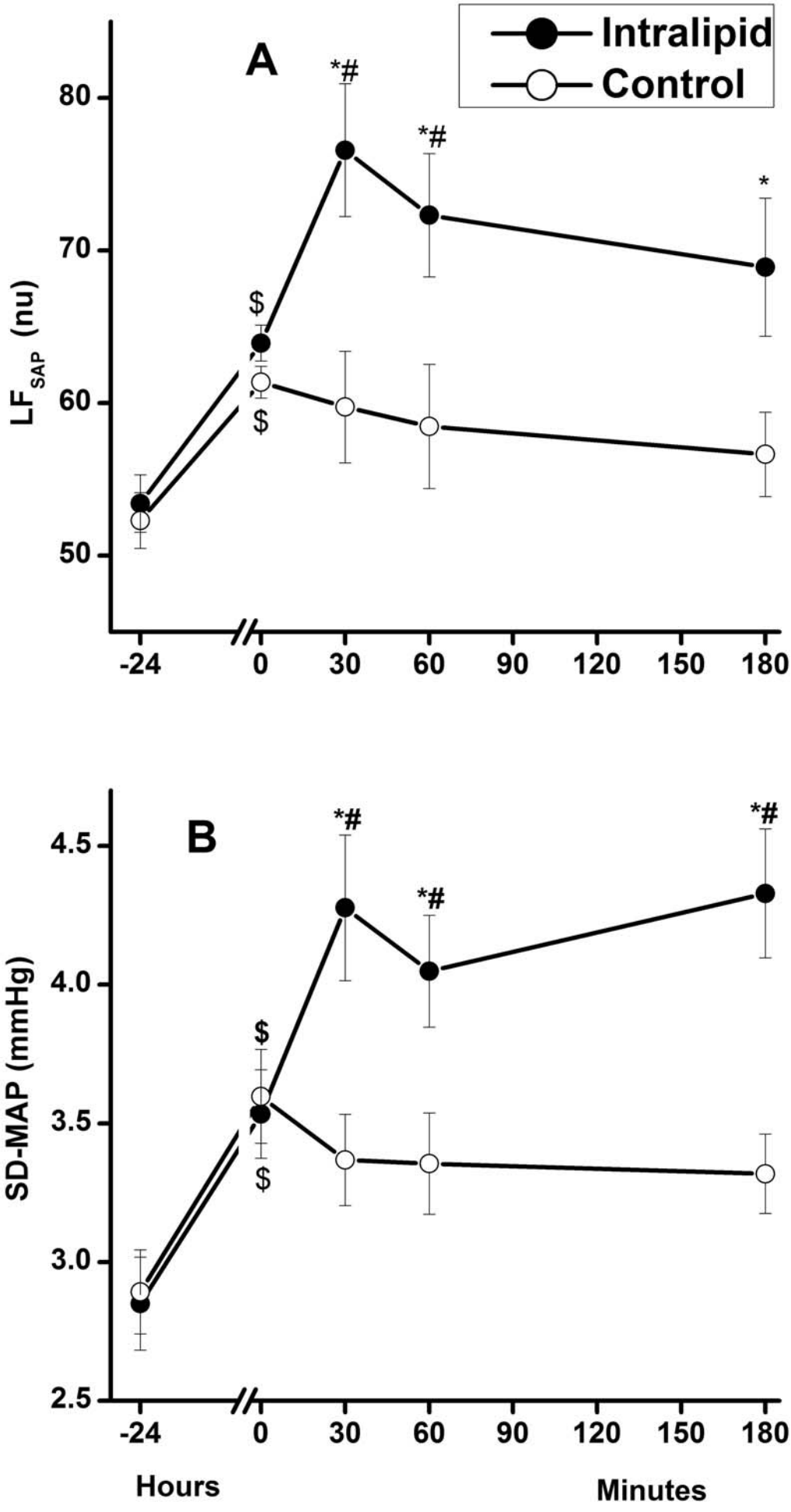

Fig. 5

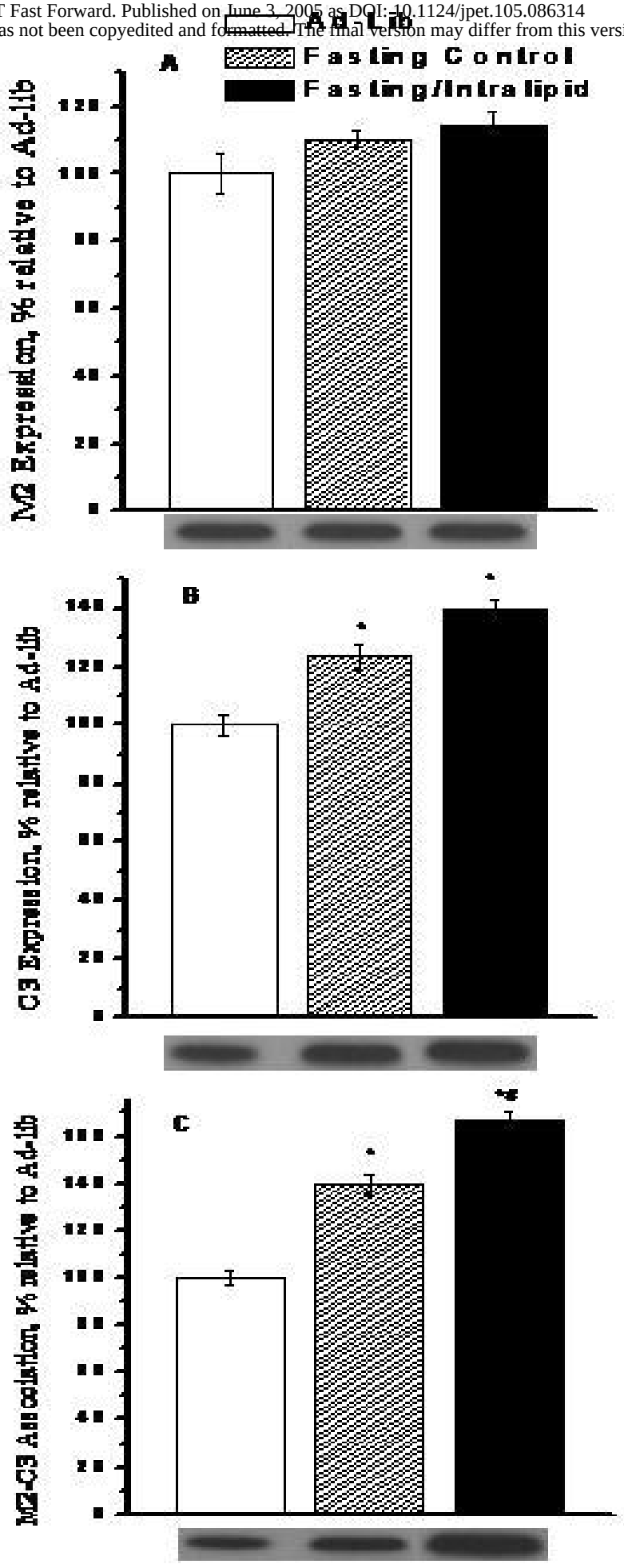

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Fig. 6