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# Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus

Cláudio T. De Souza,* Eliana P. Araujo,* Silvana Bordin, Rika Ashimine, Ricardo L. Zollner, Antonio C. Boschero, Mário J. A. Saad, and Lício A. Velloso

*Departments of Internal Medicine (C.T.D.S., E.P.A., R.A., R.L.Z., M.J.A.S., L.A.V.) and Physiology and Biophysics (A.C.B.), State University of Campinas, 13084-970 Campinas-SP; and Department of Physiology and Biophysics (S.B.), University of São Paulo, 05508-900 São Paulo-SP, Brazil*

**Obesity has reached epidemic proportions in several regions of the world. General changes in lifestyle, including consumption of fat-rich food, are among the most important factors leading to an unprecedented increase in the prevalence of this disease. Weight gain results from an imbalance between caloric intake and energy expenditure. Both of these parameters are under the tight control of specialized neurons of the hypothalamus that respond to peripheral anorexigenic and adipostatic signals carried by leptin and insulin. Here we show, by macroarray analysis, that high-fat feeding [hyperlipidic diet (HL)] induces the expression of several proinflammatory cytokines and inflammatory responsive proteins in hypothalamus. This phenomenon is accompanied by increased activation of c-Jun N-terminal kinase and nuclear factor-κB. In addition, HL feeding leads to impaired functional and molecular activation of the insulin-signaling pathway, which is paralleled by increased serine phosphorylation of the insulin receptor and insulin receptor substrate-2. Intracerebroventricular treatment of HL rats with a specific inhibitor of c-Jun N-terminal kinase (SP600125) restores insulin signaling and leads to a reduced caloric intake and weight loss. We conclude that HL feeding induces a local proinflammatory status in the hypothalamus, which results in impaired anorexigenic insulin signaling. (*Endocrinology* 146: 4192–4199, 2005)**

DURING THE LAST DECADE, great advances were made in the characterization of the role played by the hypothalamus in the coordination of food intake and energy expenditure (1, 2). Leptin and insulin were shown to provide the most robust signals to specialized neurons of the hypothalamus to inform about energy stocks in the periphery and trigger physiologic responses aimed at controlling hunger and thermogenesis (1, 3, 4). Both hormones act through receptors located, mostly, in at least two subpopulations of neurons in the arcuate nucleus of the hypothalamus. Low levels of insulin or leptin, as observed during fasting or when total body fat mass is depleted, lead to the activation of neuropeptide Y/agouti gene-related peptidergic neurons, providing stimulatory signals to melanin-concentrating hormone- and orexin-producing neurons of the lateral hypothalamus. The consequence is increased hunger and decreased energy expenditure. In contrast, after a meal or when body fat stores are replete, the levels of insulin and leptin rise, leading to inhibition of neuropeptide Y/agouti gene-related peptidergic neurons and stimulating proopiomelanocortin/cocaine- and amphetamine-induced transcriptergic neurons; in turn, a reduction of orexin and melanin-concentrating hormone occurs in the lateral hypothalamus, accompanied by increased production of CRH and TRH in the periventricular nucleus, which promotes satiety and increases thermogenesis (4). Establishing a parallel between the development of type 2 diabetes mellitus and obesity, it has been proposed that hypothalamic resistance to insulin and leptin action plays a central role in the loss of coordinated control of food intake and energy expenditure (4, 5). In some animal models, this phenomenon has been documented (6, 7), but little progress has been achieved in the characterization of mechanisms that trigger and mediate insulin and leptin resistance in the hypothalamus.

In peripheral tissues, insulin resistance develops as a consequence of several variable factors, beginning with an appropriate genetic background which must, to a certain degree, be associated with environmental variables such as ageing, feeding patterns, sedentary lifestyle, stress, and infections among others. An environmental factor strongly linked with the development of peripheral insulin resistance, type 2 diabetes, and obesity is the consumption of fat-rich diets (8).

The primary objective of the present study was to evaluate the effect of long-term consumption of hyperlipidic (HL) diet on the expression of several mRNA specificities in hypothalamus of rats. Because HL diet promoted the regulation of expression of a number of mRNAs encoding for proteins that participate in the inflammatory response, we next evaluated the role of this proinflammatory phenomenon in insulin signal transduction and feeding behavior of rats.

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* C.T.D.S. and E.P.A. contributed equally to these studies.

Abbreviations: Ctr, Control; HL, hyperlipidic; icv, intracerebroventricular; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; NFκB, nuclear factor-κB; PI3-kinase, phosphatidylinositol 3-kinase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

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## Materials and Methods

### *Experimental animals and HL diet*

Male 4-wk-old Wistar rats from the University of Campinas Breeding Center were randomly divided into two groups, control (Ctr), fed standard rodent chow *ad libitum* (protein, 20 kcal%; carbohydrate, 70 kcal%; lipid, 10 kcal%) and HL, fed a fat-rich chow *ad libitum* (protein, 20 kcal%; carbohydrate, 35 kcal%; lipid, 45 kcal%). This diet composition has been previously used (9). For macroarray analysis, hypothalami were obtained from rats of each group after 16 wk of dieting. For evaluation of cytokine expression by ELISA and Western blot and evaluation of c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NFκB) activation, hypothalami were obtained from control rats at 4 wk of age (Ctr0) or control or HL rats at 13 (Ctr13, HL13) or 16 wk (Ctr16, HL16) after beginning a specific diet. For RT-PCR evaluation of cytokine expression, immunohistochemistry, and signal transduction studies, hypothalami were obtained from control and HL rats at 16 wk (Ctr16, HL16) after beginning a specific diet. As a rule, the hypothalamic fragments used for mRNA and protein extraction were 4.0 $mm^3$ and had the optic chiasm as the rostral limit (bregma −0.25 mm), the infundibular stem as the caudal limit (bregma −4.20 mm) and were 4.0 mm wide and 3.0 mm deep. Fragments for immunohistochemical studies were larger (~8.0–9.0 $mm^3$), and the correct topographical localization of the sections was obtained elsewhere (10). In some rats the JNK-specific inhibitor SP600125 (Tocris, Ellisville, MO) was administered intracerebroventricularly (icv), twice a day, for 1 wk (from wk 15 to 16 after beginning a specific diet) (Ctr16SP and HL16SP). The doses of 12, 23, and 46 μmol/dose were tested in dose-response experiments and, because similar effects to inhibit [$Thr^{183}$] phosphorylation of JNK were obtained with 23 and 46 μmol/dose, the dose of 23 μmol/dose was used for signal transduction and functional experiments. Some rats of the HL group were submitted to 1 wk pair feeding (HL16PF) with rats treated with SP600125 (HL16SP).

### *Intracerebroventricular cannulation*

For SP600125 treatment, insulin-induced inhibition of food intake and insulin or leptin signal transduction experiments rats were stereotaxically instrumented using a Stoelning stereotaxic apparatus, according to a method previously described (6). Cannula patency was tested 1 wk after cannulation by the evaluation of the drinking response elicited by icv angiotensin II (11).

### *Body and epididymal mass, icv insulin-induced inhibition of 12-h food intake, and daily caloric intake*

All these parameters were evaluated following protocols previously described (6, 7).

### *RNA preparation for macroarray and RT-PCR*

Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's recommendations. Total RNA was rendered genomic DNA free by digestion with Rnase-free Dnase (RQ1; Promega, Madison, WI).

### *Macroarray analysis*

Rat 1.2 Atlas Array was from Clontech (Palo Alto, CA). One microgram of poly(A)$^+$ RNA was converted into $^{33}$P-labeled first strand cDNA by Moloney murine leukemia virus reverse transcriptase. Unincorporated $^{33}$P-labeled nucleotides were removed by chromatography using a NucleoSpin extraction spin column (Clontech). Purified cDNA probes were hybridized to the Atlas membranes. Hybridization occurred overnight at 68 C. After washing, membranes were sealed in hybridization bags and exposed to imaging plates for 1 d. After exposure, the imaging plates were scanned using a BAS-1500 (Fujifilm, Fuji, Japan), and hybridization signals were counted. Hybridization signals of each gene were normalized by a positive control (signals of the housekeeping gene), and gene expression was compared between the preconditioning and control groups.

### *Semiquantitative RT-PCR*

Seven micrograms of total RNA were reverse transcribed with SuperScript reverse transcriptase (200 U/μl) using oligo (dT) (50 mM) in a 30 μl reaction volume (5× reverse transcription buffer, 10 mM deoxynucleotide triphosphate, and 40 U/μl Rnase-free inhibitor). The reverse transcriptions involved a 50-min incubation at 42 C and a 15-min incubation at 70 C. The PCR products were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized by excitation under UV light. Photodocumentation was performed using the Nucleovision system (NucleoTech, San Mateo, CA), and band quantification was performed using the Gel Expert software (NucleoTech). In all samples the amplification of cyclophiline was performed and used as an internal control for quantity and quality. The semiquantitative expression of cytokines was calculated using the formula: semiquantitative expression = pixel area of product/pixel area of cyclophilin × 100. The primers used were: TNFα sense, 5′-GTG CCT CAG CCT CTT CTC ATT CC-3′, antisense, 5′-GCT CCT CCG CCT GGT GGT TT-3′ (product 218 bp); IL-1β sense, 5′-GGA TGA TGA CGA CCT GC-3′, antisense, 5′-TCC CGA CCC TTG CTG TT-3′ (product 450 bp); IL-6 sense, 5′-CCT TCT TGG GAC TGA TGT-3′, antisense, 5′-CTC TGG CTT TGT CTT TCT-3′ (product 384 bp); and cyclophilin sense, 5′-GAC AGC AGA AAA CTT TCG TGC-3′, antisense, 5′-GGT TCT GAC TCA CCG ACC T-3′ (product 276 bp).

### *ELISA for cytokine determination*

Tissue cytokine levels were determined in samples of hypothalamic protein extracts (2.0 mg/ml) by ELISA (Pierce Biotechnology, Rockford, IL), following the recommendations of the manufacturer.

### *Determination of NFκB activation*

Basal hypothalamic NFκB activation was determined in nuclear extracts by ELISA (Pierce Biotechnology) according to the recommendations of the manufacturer and using positive and negative controls supplied with the kit.

### *Tissue extraction, immunoblotting and immunoprecipitation*

The icv cannulated rats were anesthetized and acutely treated with saline (2.0 μl), insulin ($10^{-6}$ M, 2.0 μl), or leptin ($10^{-6}$ M, 2.0 μl). After 2 min [for insulin receptor (IR)], 5 min [for insulin receptor substrate (IRS)-2, phosphatidylinositol 3-kinase (PI3-kinase), and Akt], or 10 min [for signal transducer and activator of transcription (STAT)-3], the hypothalami were obtained and immediately homogenized in solubilization buffer at 4 C [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY). Insoluble material was removed by centrifugation for 20 min at 9000 × *g* in a 70. Ti rotor (Beckman, Fullerton, CA) at 4 C. The protein concentration of the supernatants was determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 2.0 mg of total protein were used for immunoprecipitation with antibodies against IR, IRS-2, and STAT-3 at 4 C overnight, followed by SDS-PAGE, transfer to nitrocellulose membranes, and blotting with antiphosphotyrosine, antiphosphoserine, anti-IR, anti-IRS-2, anti-STAT-3, or anti-p85/PI3 kinase. In direct immunoblot experiments, 0.2 mg of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-TNFα, anti-IL-1β, anti-IL-6, anti-IR, anti-IRS-2, anti-STAT-3, anti-suppressor of cytokine signaling (SOCS)-3, anti-phospho-[$Ser^{473}$]Akt, and anti-phospho-[$Thr^{183}$]-JNK antibodies, as described (12). The protein loading of immunoblots was always evaluated by two methods: before blotting, the nitrocellulose membranes were stained with Coomassie Blue, and after blotting the membranes were reprobed with an antivimentin antibody.

### *Optic and confocal microscopy*

Paraformaldehyde-fixed hypothalami were sectioned (5 μm) and used in regular single- or double-immunofluorescence staining using

TNFα, NeuN, or phospho-[Thr$^{183}$]-JNK antibodies according to a previously described protocol (6, 13). Analysis and documentation of results were performed using an LSM 510 laser confocal microscope (Zeiss, Göttingen, Germany) and a BX60 microscope (Olympus, Tokyo, Japan). Semiquantitative assessment of NeuN, TNFα and phospho-[Thr$^{183}$]-JNK was achieved by counting the number of positively stained cells. Every second of all consecutive sections were counted in arcuate nucleus and lateral hypothalamus, sites of predominant staining for both TNFα and phospho-[Thr$^{183}$]-JNK. The anatomical correlations were made according to the landmarks given in a stereotaxic atlas (10). The topographical views of the regions to be studied were obtained by hematoxylin-eosin staining of consecutive sections.

### *Statistical analysis*

The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software (Scion Corp., Frederick, MD). All data, except those from immunohistochemistry and macroarray, were analyzed by the two-tailed unpaired Student's *t* test or by repeat-measures ANOVA (one-way or two-way ANOVA) followed by *post hoc* analysis of significance (Bonferroni test) when appropriate, comparing experimental and control groups. In immunohistochemistry the average number of positive cells per section was obtained for each animal. Differences between groups were evaluated by the nonparametric Kruskal-Wallis and Mann-Whitney *U* test. The data are expressed as percent variation from respective control. In macroarray analysis, genes exhibiting differential expression in the preconditioning group were selected only if the hybridization signals were either increased or decreased by at least 2-fold, compared with those of the control group, and according to the statistical analysis performed by ANOVA. The functional clustering of genes was performed by combining the information obtained from public databases such as the National Institutes of Health (www.ncbi.nlm.nih.gov) and the Rat Genome Database (www.rgd.mcw.edu). In all experiments, the level of significance was set at $P < 0.05$.

## Results and Discussion

To evaluate the role of HL feeding on hypothalamic protein expression, Wistar rats treated during 16 wk with a diet containing 45 kcal% fat were used for macroarray analysis of 1176 mRNAs in hypothalamus in comparison with rats treated with a regular rodent chow, containing 10 kcal% fat. HL feeding modulated the expression of 170 mRNAs specificities (92 up-regulated and 78 down-regulated; complete data presented in supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), including hormones, growth factors, transcription factors, enzymes of lipid metabolism, and membrane and nuclear receptors among others. HL feeding modulated the expression of mRNAs encoding proteins known to participate in the control of feeding, thermogenesis, and other important metabolic functions. These mRNAs included the IR (5.42 ± 0.03-fold increase), leptin receptor ObR (8.89 ± 0.07-fold increase), CRH binding protein (3.52 ± 0.03-fold increase), and *N*-methyl-D-aspartate receptor 2B (3.86 ± 0.04-fold increase). All these mRNAs were functionally clustered as metabolic-related sequences, and the data are presented in supplemental Table 2. However, after complete functional clustering, we observed that immune-related proteins' mRNAs represented the largest group modulated by HL feeding. Table 1 presents all mRNA specificities that encode immune-related proteins that were modulated by HL feeding.

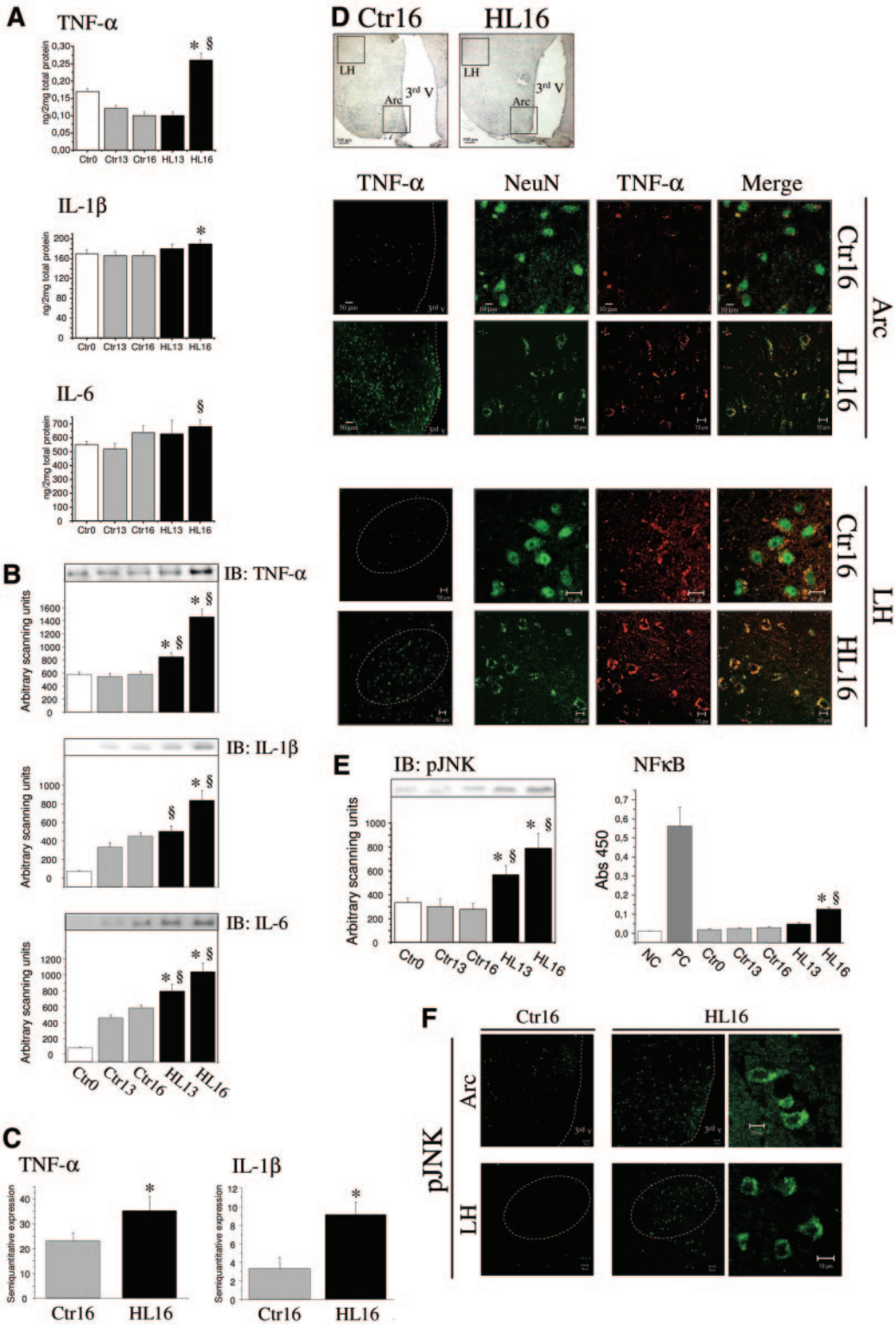
**TABLE 1.** Hypothalamic immune-related mRNA specificities modulated by high-fat feeding

| mRNA | GenBank accession no. | Fold modulation |
|---|---|---|
| 1. CD95 FAS receptor | D26112 | 2.00 ± 0.03 |
| 2. CD30L receptor | D42117 | 4.19 ± 0.03 |
| 3. CD87 monocyte activation ag | P49616 | 0.48 ± 0.03 |
| 4. CD71 transferrin receptor | M58040 | 2.05 ± 0.06 |
| 5. CD25 myelin protein | M69139 | 2.27 ± 0.03 |
| 6. NK lymph receptor | U56936 | 0.44 ± 0.08 |
| 7. IFN regulatory factor | M34253 | 2.65 ± 0.03 |
| 8. Immunoglobulin HC binding protein | M14050 | 2.12 ± 0.04 |
| 9. Fos-related ag | M19651 | 2.32 ± 0.10 |
| 10. 5-Lipoxygenase | J03960 | 0.34 ± 0.02 |
| 11. TGFβ receptor | L26110 | 2.09 ± 0.02 |
| 12. Endothelin receptor 1 | M60786 | 2.33 ± 0.05 |
| 13. Prostaglandin D2 receptor | U9289 | 2.86 ± 0.08 |
| 14. Prostaglandin E2 receptor EP2 | U94708 | 2.11 ± 0.03 |
| 15. Thromboxane A2 receptor | D21158 | 2.51 ± 0.06 |
| 16. Glucocorticoid receptor | M14053 | 2.05 ± 0.04 |
| 17. TGF-β3 | U03491 | 3.45 ± 0.04 |
| 18. TNF-α | X66539 | 4.67 ± 0.05 |
| 19. Macrophage MIP3 | U90447 | 0.33 ± 0.02 |
| 20. IL-1β | M98820 | 2.34 ± 0.06 |
| 21. IL-2 | M22899 | 2.82 ± 0.03 |
| 22. IL-6 | M26744 | 4.93 ± 0.06 |
| 23. IL-7 | AF010464 | 2.43 ± 0.03 |
| 24. JAK2 | U13396 | 0.49 ± 0.03 |
| 25. JNK3 | L27128 | 0.50 ± 0.03 |
| 26. Mast cell protease 8 | U67911 | 0.20 ± 0.01 |
| 27. Mast cell protease 7 | U67910 | 0.18 ± 0.01 |
| 28. Mast cell protease 3 | D38495 | 0.01 ± 0.00 |
| 29. IFN-induced protein | X61381 | 0.34 ± 0.01 |

Atlas Rat 1.2 Array; n = 3; fold modulation as compared to expression in hypothalamus of rats fed control diet ± SEM. IFN, Interferon; MIP, macrophage inflammatory protein; JAK, Janus kinase; NK, natural killer; HC, heavy chain.

In recent years, a bulk of evidence has been obtained to show that certain proinflammatory cytokines, produced by the adipose tissue, participate in the induction of insulin resistance in tissues that act as traditional targets for insulin action (14, 15). This phenomenon is mediated by the activation of intracellular serine kinases such as JNK and IκB kinase, which act as intermediaries for proinflammatory signaling, but are also able to induce serine phosphorylation of key elements of the insulin signal transduction pathway and by this mechanism blunt insulin action (14–16). TNFα, IL-1β, and IL-6 have been described as inducers of insulin resistance by the aforementioned mechanism (14–16). In the macroarray analysis, the mRNAs of all these three cytokines were increased by the consumption of the HL diet. Therefore, we used distinct methods to evaluate the expression/concentration of these cytokines in the hypothalamus of rats. By ELISA (Fig. 1A), it was shown that the hypothalamic levels

FIG. 1. Expression of cytokines and activation of proinflammatory signaling in hypothalamus of HL rats. A, Determination of tissue concentration of TNFα, IL-1β, and IL-6 by ELISA in hypothalamus of rats (n = 5; *, $P < 0.05$ *vs.* respective control; §, $P < 0.05$ *vs.* Ctr0). B, Evaluation of hypothalamic expression of TNFα, IL-1β, and IL-6 by immunoblot (n = 5; *, $P < 0.05$ *vs.* respective control; §, $P < 0.05$ *vs.* Ctr0). C, Evaluation of TNFα and IL-1β mRNA expression, by RT-PCR, in hypothalamus of rats (n = 5, *, $P < 0.05$). D, Evaluation of TNFα expression in arcuate nucleus (Arc) and lateral hypothalamus (LH) by double-staining immunofluorescence; neuron bodies were labeled with NeuN (figures are representative of three independent experiments); the *upper figures* are hematoxylin-eosin stainings of sequential sections and allow a




topographical view of the regions studied in D and F; approximate regions used for the studies of the arcuate nucleus and the lateral hypothalamus are marked with *squares*. E, Immunoblot evaluation of steady-state [Thr$^{183}$] phosphorylation of JNK and ELISA evaluation of steady-state NFκB activation in hypothalamic nuclear extracts of rats (in both experiments n = 5; *, $P < 0.05$ *vs.* respective control; §, $P < 0.05$ *vs.* Ctr0; in NFκB experiments NC and PC refer to negative and positive control nuclear extracts provided by the manufacturer). F, Basal expression and localization of phospho-[Thr$^{183}$]-JNK in arcuate nucleus and lateral hypothalamus of rats (figures are representative of three independent experiments). Ctr0, 4-wk-old rats fed regular chow; Ctr13 and Ctr16, rats fed regular chow for 13 or 16 wk; HL13 and HL16, rats fed fat-rich chow for 13 or 16 wk. In the immunohistochemical studies, the sections were obtained at bregma −2.5 mm according to Ref. 13.

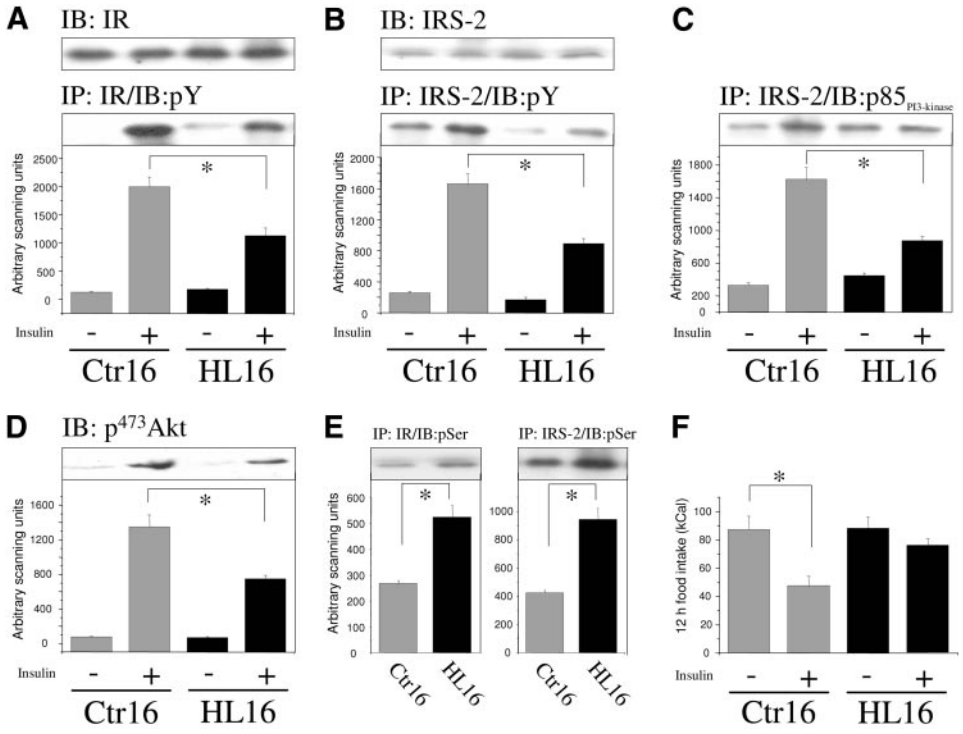



FIG. 2. Insulin signal transduction and action in hypothalamus of HL rats. Hypothalamic protein extracts containing 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with anti-IR (A, *upper blot*); anti-IRS-2 (B, *upper blot*); or antiphospho-[$Ser^{473}$]-Akt (D) antibodies. Hypothalamic protein extracts containing 2.0 mg were used in immunoprecipitation (IP) assays with anti-IR (A, *lower blot*; and E, *first blot*); or anti-IRS-2 (B, *lower blot*; C and E, *second blot*) antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antiphosphotyrosine (pY) (A and B); anti-$p85_{PI3\text{-}kinase}$ (C), and antiphosphoserine (pSer) (E) antibodies. F, After 6 h fasting, rats were treated with a single icv dose of saline (−) (2.0 $\mu$l) or insulin (+) (2.0 $\mu$l, $10^{-6}$ M), chow was reintroduced, and spontaneous food intake was measured over the next 12 h. In all experiments n = 5 (*, $P < 0.05$). Ctr16, Rats fed regular chow for 16 wk; HL16; rats fed fat-rich chow for 16 wk.

of TNF$\alpha$ and IL-1$\beta$ were significantly increased at 16 wk, compared with respective controls (Ctr16), whereas IL-6 was increased at 16 wk, compared with the basal control (Ctr0). By immunoblot (Fig. 1B), all three cytokines were shown to be significantly increased in the hypothalamus of rats fed on the HL diet for 16 wk, compared with respective controls (Ctr16) and basal controls (Ctr0). Moreover, by the same method, it was shown that the consumption of HL diet for 13 wk was also sufficient to promote a significant increase on TNF$\alpha$ and IL-6 expression (Fig. 1B). In addition, by RT-PCR (Fig. 1C), the levels of mRNAs encoding for TNF$\alpha$ and IL-1$\beta$ were found to be significantly increased.

By confocal microscopy, we observed that low levels of TNF$\alpha$ could be detected in the arcuate nucleus and lateral hypothalamus of rats fed on a regular diet (Fig. 1D). Feeding the rats with the HL diet for 16 wk promoted an increase in TNF$\alpha$ in both hypothalamic nuclei. In the arcuate nucleus and lateral hypothalamus TNF$\alpha$-specific staining was found both coinciding and not coinciding with neuron bodies. Specifically in the lateral hypothalamus, the HL diet produced a 44 ± 12% increase

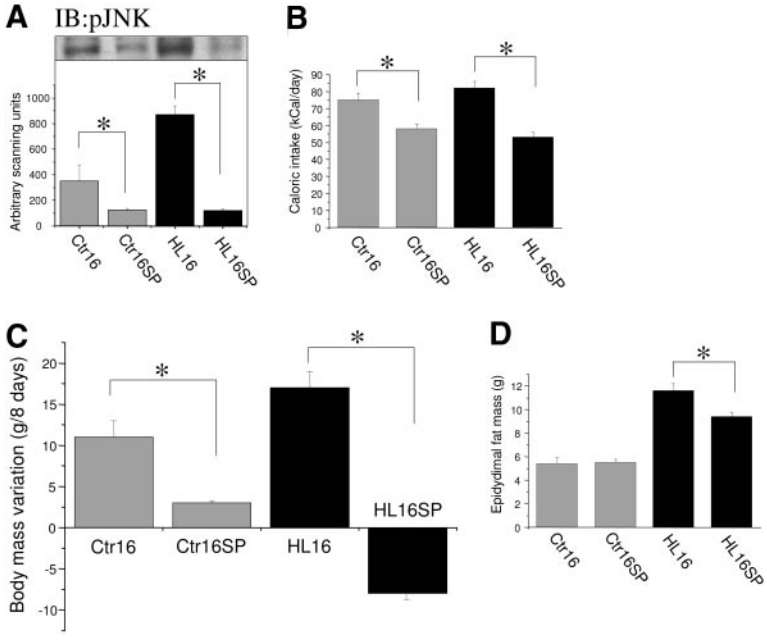



FIG. 3. Effects of SP600125 treatment on metabolic parameters of HL rats. A, Control and HL rats were icv cannulated and treated with vehicle (2.0 $\mu$l) or SP600125 (23 $\mu$mol/dose in 2.0 $\mu$l, icv) twice a day from 15th to 16th wk. Hypothalamus was obtained and samples containing 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with anti-phospho-[$Thr^{183}$]-JNK antibody. Mean daily caloric intake (kilocalories per day) (B) and body mass variation (grams per 8 d) (C) were calculated, taking into account individual 24-h food intake and body mass during the last 8 d of the experimental protocol (from 15th to 16th wk). D, Epididymal mass (grams) was determined on the last day of the experimental protocol (at the end of the 16th wk). In all experiments n = 5 (*, $P < 0.05$).

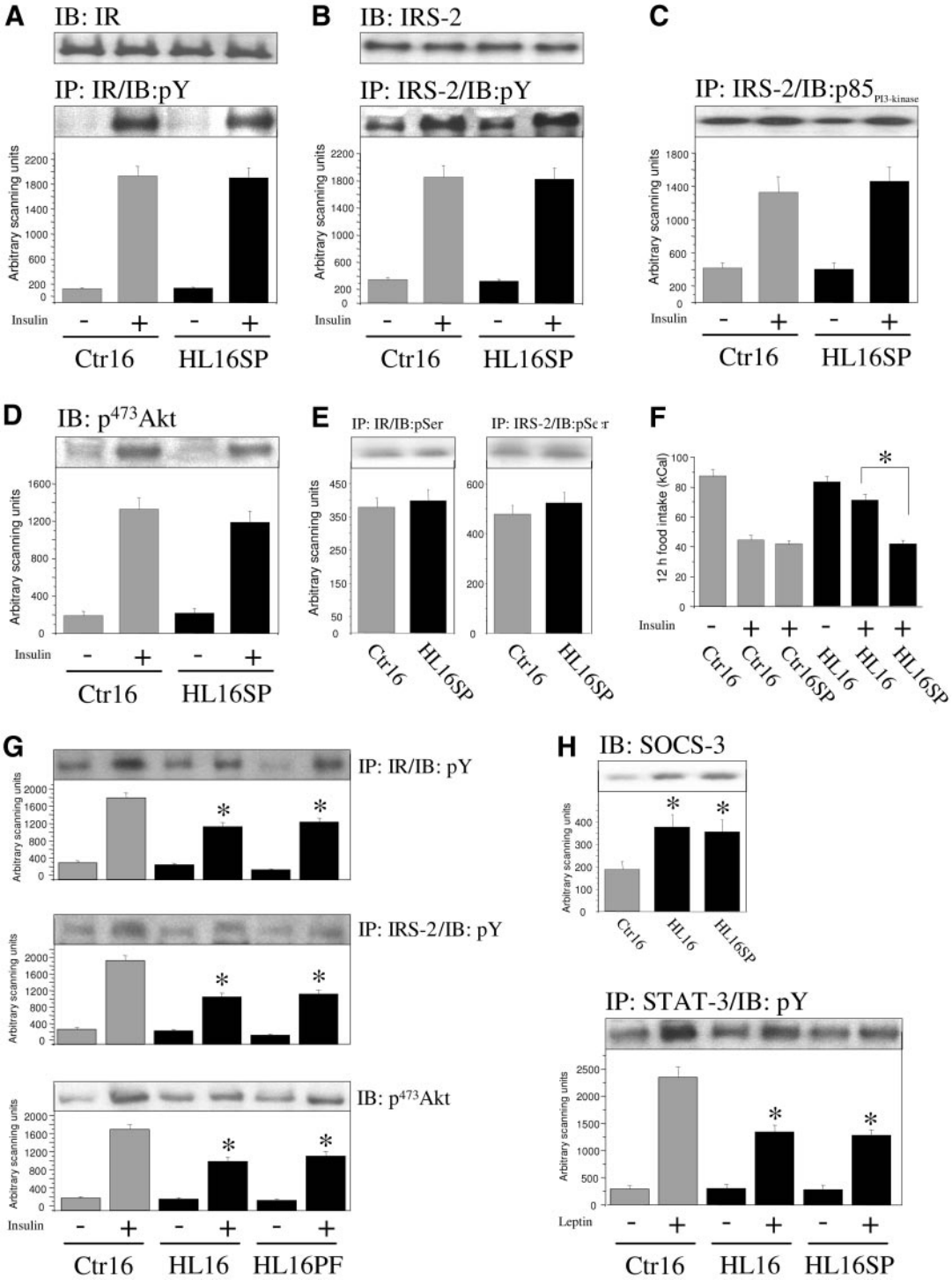



FIG. 4. Insulin and leptin signal transduction and insulin action in hypothalamus of HL rats treated with SP600125. Hypothalamic protein extracts containing 0.2 mg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with anti-IR (A, *upper blot*), anti-IRS-2 (B, *upper blot*), anti-phospho-[Ser$^{473}$]-Akt (D and G, *last blot*), or anti-SOCS-3 (H, *upper blot*) antibodies. Hypothalamic protein extracts containing 2.0 mg were used in immunoprecipitation (IP) assays with anti-IR (A, *lower blot*; E, *first blot*; and G, *first blot*), anti-IRS-2 (B, *lower blot*; C and E, *second blot*; and G, *second blot*), or anti-STAT-3 (H, *lower blot*) antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antiphosphotyrosine (pY) (A, B, and G, *first* and *second blots*; H, *lower blot*), anti-p85$_{PI3\text{-}kinase}$ (C), and antiphosphoserine (pSer) (E) antibodies. F, After 6 h fasting, rats were treated with a single icv dose of saline (−) (2.0 $\mu$l) or insulin (+) (2.0 $\mu$l, $10^{-6}$ M), chow was reintroduced and spontaneous food intake was measured over the next 12 h. F, n = 5 (*$P <$ 0.05); G and H, *, $P < 0.05$ *vs.* Ctr16. Ctr16, Rats fed regular chow for16 wk; HL16, rats fed fat-rich chow for 16 wk; HL16SP, rats fed fat-rich diet for 16 wk and treated with SP600125 (23 $\mu$mol/dose in 2 $\mu$l, icv) twice a day from 15th to 16th wk.

($P < 0.05$) in the number of TNF$\alpha$-stained neuron bodies. In other regions of the hypothalamus and the remainder of the brain, TNF$\alpha$ staining was minimal. There were no significant differences in the number of NeuN-positive cells in lateral hypothalamus and arcuate nucleus between the groups.

To evaluate whether the higher concentration of proinflammatory cytokines in the hypothalamus may lead to the activation of proinflammatory intracellular signaling, we performed immunoblots to determine phospho-[Thr$^{183}$]-JNK and ELISA to determine NF$\kappa$B activation. As depicted in Fig. 1E, the consumption of the HL diet for 13 or 16 wk significantly increased the levels of phospho-[Thr$^{183}$]-JNK, whereas the consumption of HL diet for 16 wk activated NF$\kappa$B. No phospho-[Thr$^{183}$]-JNK signal could be detected by confocal microscopy in rats fed on the control diet. However, specific staining was detected in several neuron bodies of the arcuate nucleus and lateral hypothalamus in HL rats (Fig. 1F).

The icv injection of a single dose of insulin inhibits 12 h spontaneous food intake. Most of this effect is dependent on insulin binding and activation of the IR present in neurons of the arcuate nucleus and activation of an intracellular cascade that depends on the engagement of IRS-2, PI3-kinase, and Akt (6, 7, 17). The treatment of rats with a HL diet during 16 wk did not modulate the protein expression of IR (Fig. 2A) and IRS-2 (Fig. 2B) but significantly reduced insulin-induced tyrosine phosphorylation of IR (Fig. 2A) and IRS-2 (Fig. 2B). This was accompanied by reduced insulin-induced association of IRS-2 with PI3-kinase (Fig. 2C) and reduced insulin-induced [Ser$^{473}$]-phosphorylation of Akt (Fig. 2D). Because proinflammatory signaling is known to induce molecular resistance to insulin by promoting serine phosphorylation of key elements of the insulin signaling pathway (14, 15), we performed immunoblot analysis of IR and IRS-2 immunoprecipitates obtained from hypothalamic protein extracts of rats fed on control and HL diets. As depicted in Fig. 2E, both IR and IRS-2 presented higher levels of serine phosphorylation in samples from rats fed on HL diet, compared with the control rats. Finally, insulin-induced inhibition of spontaneous food intake was severely affected by the consumption of the HL diet (Fig. 2F), demonstrating that, in parallel to the molecular resistance to insulin, high-fat feeding negatively modulates a functional phenomenon controlled by insulin in the hypothalamus.

The reason the IR protein level was not modulated by HL feeding whereas IR mRNA was significantly increased is not known. We suspect that, as in other circumstances, proinflammatory events may induce higher protein turnover and differential regulation of transcription and translation (18). In this context, a recent study has shown that the cytokine signaling suppressors, SOCS-1 and -3, direct elements of the insulin signaling pathway to proteosomic degradation, and therefore, accelerate the rate of protein turnover (19). The fact that the IR protein amount was not effectively increased by HL feeding may have implications not only in insulin signal transduction in hypothalamic neurons but also in the rate of insulin transport across the blood-brain barrier. We have not measured this transport capacity, but previous studies have shown that, in animal models of obesity and insulin resistance, the binding of insulin to brain capillaries is impaired, suggesting the participation of this mechanism in the inappropriate action of insulin in the central nervous system (20, 21).

To test the hypothesis that proinflammatory signaling stimulated by HL feeding may be involved in the inhibition of insulin signaling in hypothalamus, we treated control and HL rats with a specific inhibitor of JNK, SP600125. The compound was icv injected and after dose optimization based on previous reports (22), we observed that an almost complete inhibition of [Thr$^{183}$] phosphorylation of JNK was obtained in control and HL rats (Fig. 3A). The treatment with the compound was also able to significantly reduce daily caloric intake (Fig. 3B) and promote a loss of body mass (Fig. 3C). Mean body mass at the beginning of the period of treatment was 338 $\pm$ 24, 342 $\pm$ 26, 403 $\pm$ 20, and 405 $\pm$ 22 g for control, control treated with SP600125, HL, and HL treated with SP600125, respectively. At the end of the treatment period (8 d), the body mass was 350 $\pm$ 23, 345 $\pm$ 22, 420 $\pm$ 24, and 397 $\pm$ 19 g for control, control treated with SP600125, HL, and HL treated with SP600125, respectively. In control rats, the compound also exerted some effect promoting a significant reduction in daily caloric intake (Fig. 3B). However, this was of a significantly ($P < 0.05$) lower magnitude than in HL rats and led only to a reduction of the rate of body mass gain (Fig. 3C). Interestingly, the loss of body mass promoted by the treatment with SP600125 was, in part, due to a significant fall in intraabdominal fat, as determined by the measurement of epididymal fat mass (Fig. 3D).

Next, we evaluated the effect of icv injection of SP600125 on insulin signaling and action in the hypothalamus of rats fed on the HL diet. As depicted in Fig. 4, A and B, the compound exerted no effect on IR and IRS-2 protein expression. It did, however, revert the effect of HL feeding on insulin-induced tyrosine phosphorylation of IR and IRS-2 in such a way that no difference was found between control and rats fed on the HL diet and treated with the compound. Restoration of insulin signaling through PI3-kinase and Akt was also obtained by the icv injection of SP600125 in HL rats (Fig. 4, C and D). All these phenomena were accompanied by a reduction in Ser phosphorylation of IR and IRS-2, which returned to levels similar to those of the control (Fig. 4E). Moreover, the treatment of HL rats with SP600125 significantly increased insulin-induced inhibition of 12 h spontaneous food intake, whereas no effect of the compound was detected in control rats (Fig. 4F). To determine whether the effect of SP600125 on hypothalamic insulin signal transduction was dependent on the reduction in food intake and loss of body mass, we evaluated insulin-induced tyrosine phosphorylation of the IR and IRS-2 and [Ser $^{473}$]-phosphorylation of Akt in pair-fed, HL diet-treated rats. Pair feeding promoted a similar body mass loss to that produced by SP600125 treatment. However, as depicted in Fig. 4G, pair feeding was not enough to restore baseline insulin-induced molecular activation of these proteins. Therefore, we suspect that most of the effect of SP600125 is mediated by its specific action on JNK and not through indirect actions on food intake and body mass.

Hypercaloric and particularly hyperlipidic feeding is implicated as one of the most important environmental factors leading to obesity. Recent studies have shown that ingestion of a fat-rich diet leads to hypothalamic resistance to leptin (9, 23). This phenomenon is partially dependent on the immune-related signaling suppressor SOCS-3, which interacts with and inhibits elements of the ObR signaling pathway (24, 25).

To evaluate whether the proinflammatory phenomenon, herein described, may somehow interact and modulate canonical leptin signaling, we determined SOCS-3 expression and leptin-induced activation of STAT-3 in hypothalamus. As shown in Fig. 4 h, the consumption of the HL diet for 16 wk promoted a significant increase in the hypothalamic expression of SOCS-3, which was not reverted by a 1-wk treatment with SP600125. In addition, HL diet consumption led to the inhibition of leptin-induced tyrosine phosphorylation of STAT-3. Once more, inhibition of JNK with SP600125 did not revert the inhibitory effect of HL feeding on the ability of leptin to induce STAT-3 molecular activation.

In the present study, we demonstrate that the consumption of a fat-rich diet modulates the expression of several hypothalamic mRNAs encoding proteins with immune-related activities. The ability of certain types of fatty acids to modulate the production of cytokines has been known for quite some time and both pro- and antiinflammatory outcomes have been described, depending on the type of fatty acid studied (26, 27). Distinct mechanisms seem to participate in fatty acid-dependent regulation of the immune response. These mechanisms range from the control of prostaglandin E2 and leukotriene B4 production, which may affect signal transduction through IκB kinase (28, 29), to direct action on nuclear receptors controlling the production of immune-related factors (30). Although we cannot be sure whether the proinflammatory phenomenon, herein described, is a direct or indirect consequence of the consumption of fat-rich food, we believe our data have established an important link between a common environmental event, *i.e.* consumption of fat-rich food, and the generation of a microenvironmental phenomenon of inflammation at an anatomical site involved in the central control of metabolic functions. Moreover, this study shows that inhibition of a protein that plays a central role in one of the most important proinflammatory intracellular signaling pathways, *i.e.* JNK, improves metabolic parameters and restores hypothalamic insulin signaling in rats fed a HL diet. These data place local inflammation into a pivotal position in the pathophysiological mechanisms involved in diet-induced obesity and opens novel therapeutic perspectives for this medical condition.

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Address all correspondence and requests for reprints to: Lício A. Velloso, Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas, Campinas-SP 13083-970, Brazil. E-mail: lavelloso@fcm.unicamp.br.

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