

Vascular Expression of Proinflammatory Transcription Factors in a Model of Metabolic Syndrome

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SUMMARY

With the goal of assessing the expression of redox-sensitive transcription factors in the arteries of an experimental model of hypertension associated to metabolic syndrome (FFHR), we studied Wistar Kyoto rats (WKY) and spontaneously hypertensive 30-day old male rats (SHR), which were randomly distributed in 4 groups (n=8 in each group). Group 1: WKY (control rats), Group 2: FFR: rats that received 10% W/V fructose in drinking water during a 10-week period, Group 3: SHR rats and Group 4: FFHR: 2+3, i.e., SHR rats treated like Group 2.

Groups FFR and FFHR had HOMA (homeostasis assessment model) index and area under the curve (AUC) values in the glucose tolerance test, that were characteristic of insulin resistance. They also showed significant differences in plasma triglyceride and HDL cholesterol levels compared to controls, and increased their systolic blood pressure. Oxidative stress, as assessed by NAD(P)H oxidase activity and TBARS (thiobarbituric acid reactive substances) plasma concentration were significantly higher in FFR and FFHR groups, whereas in these same groups eNOS activity decreased markedly. Relative cardiac weight increased in FFR and FFHR groups, with a larger myocytes area in the left ventricular free wall. Sections of the left carotid artery exhibited eutrophic growth of the media layer in FFHR. Average optical density for anti-c-fos, anti-NF- κ B and anti-VCAM-1 antibodies was greater in resistance renal arteries and in the carotid artery of FFHR and FFR groups. The data confirm the findings of the pathological experimental model and suggest that oxidative stress and the subsequent activation of genes that participate in the inflammatory process are actively involved in the development of vascular remodeling.

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Key words > Remodeling - Metabolic Syndrome - Inflammation - Transcription genes

Abbreviations >

DAB	Dyaminobenzidine	NO	Nitric oxide
eNOS	Endothelial Nitric Oxide Synthase	ROS	Reactive oxygen species
HOMA	Homeostasis model assessment evaluation	TBA	Thiobarbituric acid
L/M	Lumen diameter to media thickness ratio	TBARS	Thiobarbituric acid reactive substances
NF-κB	Nuclear Factor - κ B	VCAM-1	Type 1 vascular adhesion molecule

INTRODUCTION

The complex interaction between genetic factors and environment in the development of arterial hypertension has been subject of controversy. Theoretically, in a population without associated risk factors, the levels of systolic arterial blood pressure are distributed in a Gaussian fashion, where the average is located between 100 and 120 mmHg. When the population is exposed to pro-hypertensive environmental factors, such as resistance to insulin, overweight or

dyslipemia, it is expected that the normal distribution curve be displaced to the right, increasing its variation, decreasing its kurtosis, and placing the mean pressure in more elevated values, even higher than the limits used in clinical diagnosis. In this type of population, the prevalence of arterial hypertension is even higher (1).

There are clear evidences on the interaction between metabolic dysfunctions and arterial hypertension. Epidemiological studies point out that more than 40% of diabetic patients are hypertensive. Hu et al

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(2) showed that the risk of having cardiovascular disease and stroke is almost 10 times higher in severe hypertensive and diabetic subjects.

Spontaneously hypertensive rats (SHR) provide a model of genetic hypertension that allows studying essential hypertension. On the other hand, the administration of carbohydrates-rich diets to rats is able to induce insulin resistance, hyperinsulinemic blood level, dyslipemia, and hypertension (3, 4). SHR which receive fructose chronically provide an experimental model (FFHR) useful to analyze the factors that contribute to the development of the metabolic syndrome. Studies on this experimental model would allow evaluating what results from the interaction of genetic and environmental factors, which is a common circumstance in daily practice, in order to interpret experimental results and explain the probable implied pathophysiological mechanisms.(5)

Different cytokines, growth factors, and adhesion molecules are involved in the development and progression of the metabolic syndrome and arterial hypertension. The increase of free fatty acids, hyperglycemia, and hyperinsulinism increase the activation of the nuclear factor κ B (NF- κ B), which in turn modulates the transcription of several cytokines and proteins related to inflammation and oxidative stress, causing the clinical expressions of the metabolic syndrome (6). Also, NF- κ B can be activated by oxygen reactive species (7). One of the regions that promote transcriptional activation of the target genes of NF- κ B is type-1 vascular cell adhesion molecule (VCAM-1). (8)

Also described is the presence of vascular structural changes associated to hypertension, phenomenon known as "vascular remodeling" (9). Morphometric studies on histological kidney slices of the media layer in SHR resistance arteries compared to their normotensive controls, and in rats that received fructose 10% in tap water (FFR) compared to their healthy controls (10,11) showed a decrease in the lumen diameter to media thickness ratio (L/M).

The objective of this study was to examine the expression of transcription factors sensitive to redox in different bed vessels of the FFHR model of genetic hypertension associated to metabolic syndrome.

MATERIAL AND METHODS

Treatment with experimental animals was carried out according to institutional guidelines. Thirty days old male Wistar Kyoto rats (WKY) and SHR were randomly distributed in four groups (n= 8 in each one) and were treated over a period of 10 weeks.

The experimental design included:

- Group 1, WKY: controls.
- Group 2, FFR: WKY rats with 10% fructose W/V administration in drinking water during the whole protocol.
- Group 3, SHR: spontaneously hypertensive rats.
- Group 4, FFHR: idem 3 + 2.

Systolic blood pressure determination

Systolic arterial pressure was assessed throughout the experimental period by plethysmographic method at the tail artery and its value was recorded with a Grass model 7 (Quincy, MA) polygraph, with previous adjustment of the animals to a 32 °C temperature environment.

HOMA Index calculation

Blood samples were drawn and heparinized plasma was separated. Baseline insulin was assessed by automatized chemiluminescence system ACS: 180 SE (Bayer, Germany). Baseline glycemia was assessed by glucose oxidase-peroxidase photolorimetric method (Wiener Lab. Rosario, Argentina). The model of homeostasis assessment (HOMA) was used as index to measure degree of insulin resistance and was calculated with the following formula: $[\text{insulin } (\mu\text{U/ml}) \times \text{glycemia } (\text{mmol/L}) / 22.5]$.

Glucose tolerance test

Three days before finalizing the experimental period, the fasting animals received an interperitoneal charge of glucose (2g/kg) and blood samples were drawn at 0, 30, 60 and 90 minutes after the charge to determine glycemia and later the area under the glycemia curve to estimate tolerance to glucose overload, expressed as mmol/L/90 min.

Assessment of the lipid profile

At the end of the experimental period, blood samples were drawn from the animals, with a previous fasting of 12 hours. Total plasma cholesterol, HDL cholesterol and triglycerides were assessed using photolorimetric enzymatic methods (Wiener Lab., Rosario, Argentina). Data are expressed in mg/dL.

Assessment of plasma lipid peroxidation

Lipid peroxidation, which is the consequence of oxidative stress, was assessed by determining the formation of thiobarbituric acid reactive substances (TBARS), measured as equivalents of malondialdehyde ($\mu\text{mol/L}$). The previously described method (15), is based on the reaction between plasma malondialdehyde, one of the products of lipid peroxidation, and thiobarbituric acid (TBA). Results are expressed in TBARS ($\mu\text{mol/L}$).

Aortic tissue NAD(P)H oxidase activity

Segments of the animals' abdominal aorta were dissected immediately after the sacrifice, they were incubated in Jude-Krebs buffer and later the enzyme activity was estimated by means of bNADPH as substrate, via superoxide production, and the ulterior emission of chemiluminescence by reaction with lucigenine. Activity was adjusted to the examined tissue and expressed as counts/min/mg.

eNOS activity in homogenates of cardiac and arterial tissue

The activity of endothelial nitric oxide synthase, Ca^{2+} /calmoduline (eNOS) dependent was measured in homogenates of mesenteric arteries (resistance bed vessel) and in left ventricle cardiac tissue, by conversion of L-[^3H]arginine in L-[^3H]citruline, as previously described. (7) Briefly, the mesenteric vessels were homogenised in a buffer solution (pH 7,4) with protease inhibitors, maintaining the samples on ice, during four intervals of 15 seconds with a Polytron homogenizer and then sonicated. Cardiac tissue was processed in a similar fashion. Following homogenates centrifugation (100 g, 5 min, 4 °C) and the assessment of

protein content (Bradford method), aliquots from the homogenates were incubated with a reaction mixture with L-[³H] arginine and the necessary cofactors for the formation of nitric oxide (NO) by eNOS, in the presence or absence of Ca²⁺/calmoduline. The reaction was stopped and the mixture was applied to a chromatography column of anionic exchange with contents of Dowex AG 50W-X8 resin and eluted with 2 mL of distilled water. The specific concentration of eluted L-[³H]citruiline was assessed by liquid scintillation counter. Calcium dependent NOS activity was calculated as the difference between activities in the presence or absence of Ca²⁺/calmoduline. Values were corrected according to protein contents in the homogenates and incubation time and are expressed as dpm/mg protein/min. The material obtained from each animal was processed independently.

Arterial structure

Changes in the structure of the arterial walls were assessed by measuring the mean layer in the left internal carotid. The organs or dissected arteries were fixed in 10% formaldehyde, were dehydrated, embedded in paraffin, and later cut in microtome. The slices were dyed with Masson trichrome solution and the diameters of the vascular wall were examined and visualized in optical microscope with 200x enhancement. Images were digitalized with Image Pro software. The linear or superficial lumen/mean (L/M) relation was calculated for each vessel with Scion Image software. Measurements of 40 slices per each arterial type of the same rat were processed, and then averaged. This result was included later in the analysis as each animal representative value.

Immunohistochemistry

The organs or dissected arteries were fixated in 10% formaldehyde, dehydrated, embedded in paraffin, and subsequently cut in microtome. They were dehydrated later, and a protocol of antigenic recovery with sodium citrate buffer was carried out. Kidney and carotid arteries histological slices were incubated with anti-c-Fos primary antibody. (Santa Cruz Biotechnology, USA), anti-NF- κ B [Subunit p65 (RelA)], (Chemicon International, USA) and anti-VCAM-1 [VCAM-1 (C-19)] (Santa Cruz Biotechnology, USA) and the respective biotinylated secondary antibodies (DAKO Cytomation, USA). Subsequent development was carried out using the ABCComplex (DAKO Cytomation, USA) technique with nickel-diaminobenzidine (DAB) (Sigma, USA) as chromogen. Results are expressed as arbitrary densitometry units.

Statistical analysis

Data, contrasted by ANOVA and Bonferroni post test, are expressed as mean \pm s.e.m. and $p < 0.05$ values were considered statistically significant.

RESULTS

As shown in Table 1, animals in all the experimental groups showed a HOMA index significantly higher than those of the control group. This variable reached its greater magnitude in the FFHR group. Values of the area under the curve in the intraperitoneal glucose overload tolerance test showed a similar pattern than HOMA index, which allowed to categorize the FFR and FFHR groups as insulin resistant.

On the other hand, the animals in the FFR and FFHR groups also showed significant differences in the levels of triglycerides and HDL cholesterol in regards to their controls. In the animals of the FFR experimental group, the levels of systolic blood pressure increased slowly throughout the whole experimental period and reached significant differences in regards to the control group at the end of the protocol. In the animals of the SHR and FFHR experimental groups were also significantly increased as from the second week of the protocol, with a slightly greater increase in the FFHR group at the end of the experimental period (Table 1).

These data, considered globally, form the clinical diagnostic criteria for the metabolic syndrome according to the Third Report of the Expert Panel ATP III (12).

The experiments performed with the objective of determining the status of oxidative stress showed that the production of superoxide due to aortic NAD(P)H oxidase enzyme activity was significantly higher in the FFR and FFHR groups (Table 1). The effect of the increased production of reactive oxygen species (ROS) is reflected in the plasma levels of peroxidized lipids. Animals in the FFR and FFHR groups significantly increased their plasma TBARS levels. Table 1 also shows the activity levels of the eNOS isoform. The generation of NO, which is a potent vasodilator and inhibitor of smooth muscle cell proliferation that form the media layer, is observed significantly decreased in the animals from the experimental group. The group of results allows describing a disorder in redox balance at vascular level, with less NO production and increased ROS production.

In regards to the variables that indicate left ventricular hypertrophy, possibly caused by post-load enhance and growth factors, it is observed that relative cardiac weight is significantly increased in the FFR group and even more in the FFHR group (Table 1). To confirm these data, the assessment of myocytes area at the ventricular free wall was performed. Table 1 shows that the increase in the area of the FFHR group is significant in regards to its controls SHR and WKY.

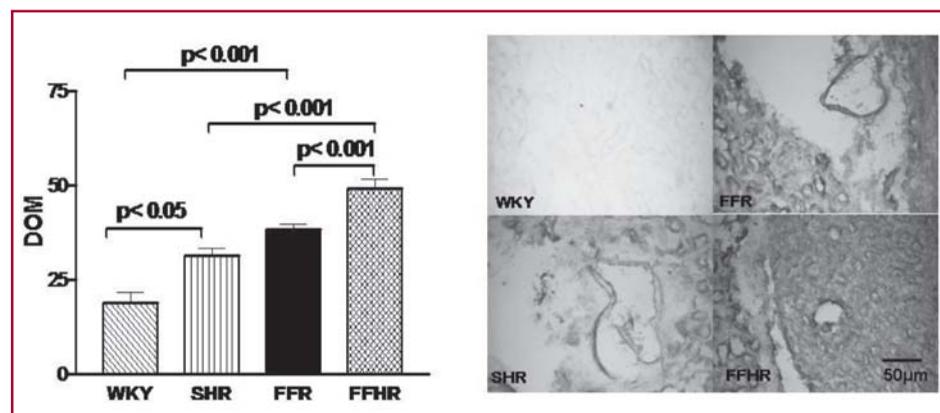
To detect the existence of changes at vascular level similar to those observed in the left ventricle, slices of the left carotid artery were examined, which showed eutrophic type growth in its media layer (Table 1). The width was significantly greater in the studied group (FFHR) and also in the FFR and SHR groups. As shown in Figure 1, the average optical density for the anti-c-Fos antibody at the resistance renal arteries of the FFHR group was significantly increased in regards to its control groups SHR and WKY, whereas it was increased in the FFR group compared to its control group (WKY). In the right panel representative microphotographs of each group are shown. It can be observed in them that the distribution of the DAB marker is similar in all groups.

Table 1. Haemodynamic, metabolic, and structural variables

Variables	WKY	SHR	FFR	FFHR
Systolic blood pressure (mm Hg)	120 ± 1,3	177 ± 0,95*	145 ± 3*	181 ± 0,95*†
HOMA Index	4,32 ± 0,1	7,20 ± 0,09*	11,93 ± 0,07*†	14,1 ± 0,45 *††
Triglycerides (mg/dl)	67,3 ± 2,6	64,3 ± 3,8	163,4 ± 3,1*†	170 ± 5,4*†
HDL Cholesterol (mg/dl)	23,5 ± 0,7	19,3 ± 0,9*	12,2 ± 0,8*†	13,6 ± 1,2*†
NAD(P)H oxidase activity (counts/min/mg tissue)	40,5 ± 6,0	133,6 ± 5,0*	155,4 ± 9,4*	665 ± 26,5*††
Arterial eNOS activity (dpm/mg prot/min)	84,3 ± 1,9	80,2 ± 2,6	59,8 ± 2,0*†	56,4 ± 5,9*†
Relative heart weight (mg/100 g)	255 ± 6	350 ± 2*	310 ± 4*	400 ± 5*††
Cardiomyocytes area (mm ²)	1.682 ± 69	2.000 ± 57	2.252 ± 180*	3.070 ± 59*††
Width of the carotid artery mean layer (µm)	45,5 ± 1,0	53,9 ± 1,4*	78,0 ± 1,5*†	90,0 ± 1,7*††

The above values correspond to haemodynamic, metabolic and structural variables. Symbols indicate: * $p < 0,01$ versus WKY; † $p < 0,01$ versus SHR; †† $p < 0,01$ versus FFR.

Fig. 1. c-Fos expression in resistance renal arteries by immunohistochemistry. The bar graphic in the left panel shows the average values and standard error in the studied groups expressed as optical density arbitrary values. To the right, representative microphotographs developed with DAB-nickel 200x.



The assessment of another activated intracellular marking in the inflammatory reaction was performed through the expression NF- κ B, as from an antibody against the p65 fraction of the p65-p50 dimer. As shown in Figure 2, the average optical density was significantly increased in the resistance renal arteries of the FFHR and FFR in regards to its control groups, but the distribution of the DAB marking, as shown in the right panel, shows that in the FFHR group this was found in the endothelium and the adventitia, whereas in the FFR group it was purely endothelial.

As from these results, we studied the expression of one of the post transcriptional products of these transcription factors that actively participates in vascular inflammation, the VCAM-1. As shown in Figure 3, the expression of VCAM-1 in the renal arteries of the FFHR and FFR groups significantly increased in comparison to its controls and with a similar distribution, not only in small caliber vessels, but also in the renal tubules. At the right, representative microphotographs of each group can be observed.

The expression of NF- κ B in the carotid arteries, as shown in Figure 4, was significantly increased in the FFHR and FFR groups in regards to its controls, but the distribution of the DAB marker, as shown in the right panel, shows that in the FFHR group this could be found in all the layers of the arterial wall, which makes it distinctive compared to the other groups.

CONCLUSIONS

The best finding in this study is the description of vascular inflammation associated to renal arterial and carotid remodeling in the experimental models that received fructose. Arteries are capable of modifying their structure and function in response to changes in haemodynamic conditions. This remodeling is characterized by hypertrophy of the wall and reduction in the L/M ratio, mediated by the synthesis and release of vasoactive and growth factors, which is associated to decreased arterial distensibility.

Folkow et al suggested that vascular remodeling is caused by sustained arterial hypertension, and this

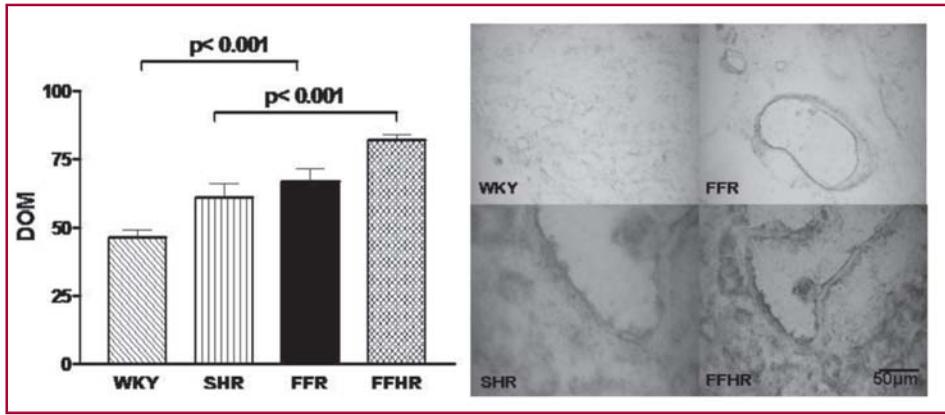


Fig. 2. p-65 fraction expression of NF-κB in resistance renal arteries by immunohistochemistry: the bar graphic in the left panel shows the average values and standard error in the studied groups, expressed as optical density arbitrary values. To the right, representative microphotographs developed with DAB-nickel 200 x.

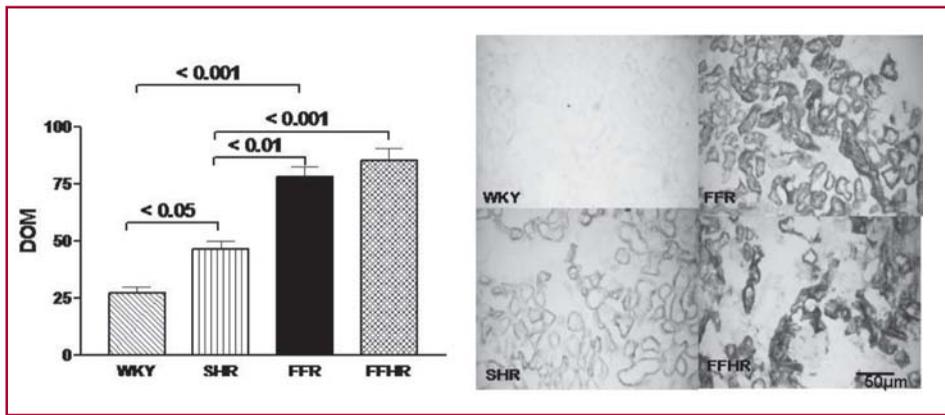


Fig. 3. VCAM-1 expression in resistance renal arteries by immunohistochemistry: the bar graphic in the left panel shows the average values and standard error in the studied groups, expressed as optical density arbitrary values. To the right, representative microphotographs developed with DAB-nickel 200 x.

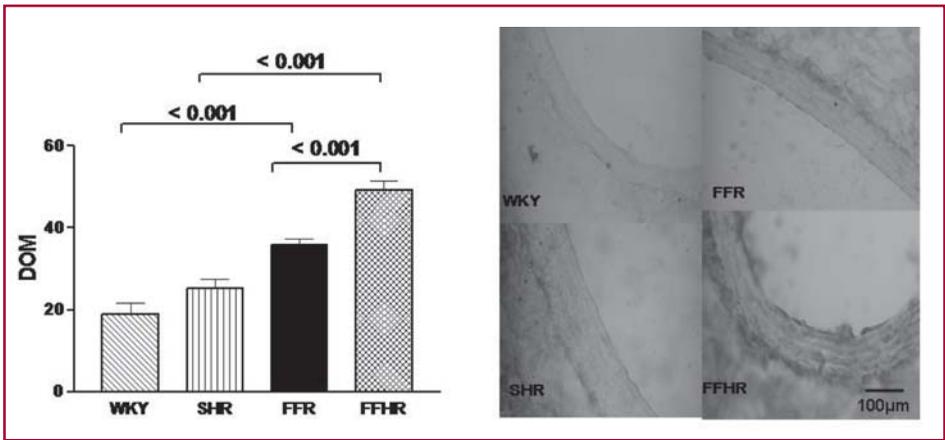


Fig. 4. p-65 of NF-κB fraction expression in carotid arteries by immunohistochemistry. The bar graphic in the left panel shows the average values and standard error in the studied groups, expressed as optical density arbitrary values. To the right, representative microphotographs developed with DAB-nickel 100x.

mechanism reinforces or secondary maintains hypertension (14). Other authors as Fever et al suggested that in some cases, the vascular hypertrophy mechanism is primary, leading to increased peripheral resistance, and consequently to hypertension (15). In this experimental model both hypothesis could co-exist: increased cascade of growth factors, product of insulin resistance and inflammation, added to hypertrophy caused by sustained hypertension.

Increased superoxide production in this model, evident by the increased lipid peroxidation, can ac-

tivate redox sensitive genes. Among them, the NF-κB can be found, which when parting the cytoplasmatic I-κB binding, this fraction migrates to the nucleus to bind to specific promoting zones. Therefore, this phenomenon could be associated with the increased expression of adhesion molecules, such as VCAM-1, which is also confirmed in this model. In addition, as described in the literature for other experimental models, an increase was found in the expression of inflammatory molecules in the vascular wall of resistance arteries which would participate in the process of vascular remodeling (16).

The potential importance of the vascular wall inflammation as a therapeutic objective continues to be an area that has not been exhaustively explored, where the new developments on the participation of the inflammatory mediators in vascular remodeling could be relevant.

In this model we observe that fructose administration induced the characteristics of metabolic syndrome and cardiovascular changes such as increased systolic pressure, cardiac hypertrophy and vascular remodeling associated with expression of inflammatory molecules. In addition, increased NAD(P)H oxidase and TBARS indicate, respectively, an increase in ROS production capability and consequently, lipidic peroxidation augmented.

Data confirm the development of the pathological experimental model and suggest that oxidative stress, and the consequent activation of genes that are involved - to some degree- in the inflammatory process, actively participate in the development of vascular remodeling.

RESUMEN

Expresión vascular de factores de transcripción proinflamatorios en un modelo de síndrome metabólico

Con el objetivo de estudiar la expresión de factores de transcripción sensibles a redox en las arterias en un modelo experimental de hipertensión arterial asociada con síndrome metabólico (FFHR), ratas Wistar Kyoto (WKY) y espontáneamente hipertensas (SHR) macho de 30 días fueron distribuidas en forma aleatoria en cuatro grupos (n = 8 c/u): 1) WKY (control), 2) FFR: administración de fructosa 10% P/V en el agua de bebida durante un período de 10 semanas, 3) SHR y 4) FFHR: ídem 3 + 2.

Los grupos FFR y FFHR presentaron valores de HOMA y área bajo la curva en la prueba de tolerancia característicos de resistencia a la insulina. También mostraron diferencias significativas en los niveles de triglicéridos y colesterol HDL respecto de sus controles y aumentaron su presión arterial sistólica. El estado de estrés oxidativo, demostrado por la actividad de NAD(P)H oxidasa y TBARS fue significativamente mayor en FFR y FFHR, en tanto que en estos mismos grupos disminuyó significativamente la actividad de eNOS. El peso cardíaco relativo aumentó en FFR y FFHR, con mayor área de los miocitos de la pared libre ventricular. Los cortes de arteria carótida izquierda mostraron crecimiento de su capa media de tipo eutrófico en FFHR. La densidad óptica media para los anticuerpos anti-c-fos, anti-NF-κB y anti-VCAM-1 fue mayor en las arterias renales de resistencia y en la carótida de los grupos FFHR y FFR.

Los datos confirman el desarrollo del modelo experimental patológico y sugieren que el estrés oxidativo y la consecuen-

te activación de genes que participan en el proceso inflamatorio intervienen activamente en el desarrollo de remodelación vascular.

Palabras clave > Remodelación - Síndrome Metabólico - Inflamación - Transcripción genética

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