Impaired Atrial M₂-Cholinoceptor Function in Obesity-Related Hypertension

Michel Pelat, Patrick Verwaerde, Christelle Merial, Jean Galitzky, Michel Berlan, Jean-Louis Montastruc, Jean-Michel Senard

Abstract—The aim of this study was to investigate the activity of the parasympathetic limb of the baroreflex arch in a canine model of obesity-related hypertension. Twelve male beagle dogs were randomized into 2 groups. Six dogs were fed with normal canine food and 6 were submitted to a 10-week high-fat diet (HFD). We have evaluated the consequences of HFD on heart rate (HR) and blood pressure (BP) circadian cycles and methylscopolamine dose-response curves. Binding of [³H]-AF-DX 384 and adenylyl cyclase activity were investigated to determine the density and functionality of M₂-cholinoceptors on right atrial membranes from control and HFD dogs. HFD induced a significant increase in body weight (15 ± 1 vs 12 ± 1 kg), systolic BP (161 ± 5 vs 145 ± 4 mm Hg), diastolic BP (92 ± 3 vs 79 ± 2 mm Hg), and HR (96 ± 4 vs 81 ± 3 bpm). Circadian rhythms of HR and BP observed in the baseline period were abolished after 9 weeks of HFD. After propranolol (1 mg/kg) pretreatment, the dose of methylscopolamine able to induce 50% maximum tachycardia was significantly increased after 9 weeks of HFD (7.4 ± 0.3 vs 4.7 ± 0.1 μg/kg). In the control group, the experimental period failed to modify these parameters. The numbers of M₂-cholinoceptors measured in right atrial membranes were significantly lower in HFD than in control groups (54 ± 6 vs 27 ± 6 fmol/mg protein). The ability of carbachol to inhibit isoproterenol-stimulated adenylyl cyclase activity was significantly lower in HFD than in control groups (IC₅₀ = 47 ± 12 vs 6.4 ± 1.4 μmol/L). However, the basal activity of adenylyl cyclase was unchanged by HFD. HFD decreases M₂-cholinoceptor number and function in cardiomyocytes. This could explain the abolition of circadian rhythm of HR and the changes in chronotropic effect brought about by methylscopolamine. (Hypertension. 1999;34:1066-1072.)

Key Words: obesity ■ receptors, muscarinic ■ diet ■ adenylyl cyclase

Epidemiological, clinical, and experimental data have established a clear relation between obesity and arterial hypertension. However, because of the methodological limitations of human studies, the nature of the pathophysiological mechanisms of the blood pressure (BP) increment still has not been elucidated. Nevertheless, some studies suggest an involvement of insulin resistance as well as sympathetic nervous activity in obesity-related hypertension. In a model obtained with the use of a high-fat diet (HFD), various abnormalities have been reported that can explain the increase in BP, such as renal or autonomic nervous system dysfunctions. As observed in obese humans, some authors have reported an increase in sympathetic nervous activity during weight gain in dogs. Moreover, a decrease in the parasympathetic drive to the heart has been reported in obese humans and in obese dogs. A recent study from our group has investigated time-course changes of autonomic nervous activity in this model and showed that parasympathetic tone is reduced in the steady state of obesity-related arterial hypertension. Nevertheless, the nature and the consequences of cardiac muscarinic cholinoceptor changes in obesity-related hypertension remain unknown.

The aim of the present study was to investigate the activity of the parasympathetic limb of the baroreflex arch in an experimental model of dogs made obese and hypertensive by a long period of an ad libitum HFD. For this purpose, we have investigated in vivo the evolution of circadian rhythm of heart rate (HR) and the response to different doses of methylscopolamine (a peripheral muscarinic cholinoceptor antagonist). Concurrently, we have performed an in vitro approach to evaluate the density of atrial M₂-cholinoceptors and their ability to modulate adenylyl cyclase activity.

Methods

Animals and General Procedure

Twelve adult beagle dogs initially weighing 10 to 12 kg were included in this study. They were all submitted to a light-dark cycle of 12 hours. A totally implantable telemetry measurement system (Data Science International) that allows continuous and long-term recording of BP and HR was surgically installed under general anesthesia.

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From Laboratoire de Pharmacologie Médicale et Clinique, INSERM U317, Faculté de Médecine, 37 allées Jules Guesde 31073 Toulouse Cedex, France.
Correspondence to Michel Pelat, Laboratoire de Pharmacologie Médicale et Clinique, INSERM U317, Faculté de Médecine, 37 allées Jules Guesde 31073 Toulouse Cedex, France. E-mail pharmed@cict.fr
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anesthesia (1 mg/kg IM acepromazine plus 10 mg/kg IV tiletamine-zolazepam) before the beginning of the study. After a recovery period (3 weeks), the dogs were randomized into 2 different groups. The control group (n = 6) was fed with 19.5 ± 2.2 g/kg of normal canine diet (Royal Canin M25®) during the entire experimental period. After the baseline period (3 weeks), the second group (HFD group, n = 6) was fed ad libitum with a HFD for 10 weeks. As previously described, 3 the hypercaloric hyperlipidic diet (containing ~42% of saturated fatty acids longer than C16) was a normal canine diet (19.5 ± 2.2 g/kg) mixed with uncooked beef fat. Body weight was measured twice per week during the entire study. All animal procedures were performed in accordance with the official regulations of the French Ministry of Agriculture.

BP and HR Measurements
In individual cages, a receiver unit (RL200, Data Science International) was used to detect and amplify the signal from each implantable transmitter and convert it into a series of digital pulses to be decoded and evaluated by computer. 18 Systolic and diastolic BPs and HR were obtained from the femoral artery pressure waveform. BP and HR signals were digitalized at 500 Hz. Systolic and diastolic BPs and HR were computed for each cycle and extracted at 2 Hz, then stored in a compatible IBM-PC for further analysis. BP and HR measurements were performed twice per week, between 9 and 10 AM, on quiet, unrestrained animals in their cages.

Circadian Rhythm of BP and HR
In control and HFD groups, BP and HR signals were continuously recorded for 24 hours at the end of the baseline period and after 9 weeks of the experimental period. To study the circadian cycle of BP and HR, 2 periods were chosen (day from 6 AM to 7 PM and night from 11 PM to 6 AM). Mean values of BP and HR were calculated for each 60-minute sequence of recording. During the entire recording period, the animals remained quiet and unrestrained in their cages.

Methylscopolamine Dose Response
To investigate the in vivo function of heart muscarinic cholinoreceptors, a dose-response experiment was performed with different doses of methylscopolamine (a peripheral nonselective muscarinic antagonist, ie, inducing an increase in HR). This experiment was performed before and after 1, 3, 5, 7, and 9 weeks of HFD and during the baseline period and after 9 weeks of HFD and control groups, respectively.

Fifteen minutes before the first methylscopolamine injection, a blockade of sympathetic endogenous stimulation of the heart with propranolol (1 mg/kg IV) pretreatment was performed in all animals. After stabilization of the HR, 6 cumulative doses (2.5, 5, 7.5, 10, 20, and 30 µg/kg IV) of methylscopolamine were administered, and HR was continuously recorded. Methylscopolamine dose-response curves were analyzed to determine maximal tachycardia (E∞) and ED50 (dose of methylscopolamine able to induce 50% of the maximal effect).

Binding Cardiomyocyte Studies
Preparation of Right Atrial Membranes
At the end of the experimental period (10 weeks after the baseline period), right atrial tissue was obtained from both control and HFD groups. Tissues were minced with scissors and homogenized in 10 volumes of ice-cold buffer (Tris-HCl 5 mMol/L, EDTA 5 mMol/L, pH 7.4) with an Ultra Turrax for 10 seconds at 24 000 rpm and 20 seconds at 12 000 rpm at 1-minute intervals in an ice-cold tube. The homogenate was diluted in the same buffer and centrifuged at 100g for 15 minutes at 4°C. The supernatant was collected and centrifuged at 20 000 rpm for 15 minutes. The final pellet was resuspended and centrifuged at 20 000 rpm for 15 minutes in ice-cold incubation buffer (Tris-HCl 50 mMol/L, MgCl2, 0.5 mMol/L, pH 7.4). The resulting pellet was finally resuspended in the required volume of incubation buffer and used immediately. Binding studies were conducted in a final volume of 400 µL. Protein content was determined by the Bradford method with bovine serum albumin as standard. 19

Saturation Experiment
As previously described by Caulfield and Birdsall, 20 determination of the number of M2-cholinoreceptors in atrial membranes was performed with the use of 2,3-dipropylamino-3H ([3H]-AF-DX 384), a specific antagonist of the M2–cholinoreceptor subtype. Membranes were incubated in the presence of 8 increasing concentrations of [3H]-AF-DX 384 ranging from 0.125 to 6 nMol/L for 30 minutes. Nonspecific binding of [3H]-AF-DX 384 was defined as the radioactivity bound to membranes that was not displaced by a high concentration (10 µMol/L) of atropine. At the end of incubation, the reaction was stopped by the addition of 4 mL of ice-cold incubation buffer followed by rapid filtration under reduced pressure through Whatmann GF/C glass fiber filters placed on a Millipore manifold. The filters were then washed twice with 10-ML portions of ice-cold incubation buffer. The radioactivity retained on the filters was measured in the presence of liquid scintillation medium (Emulsifier Safe, Packard) with the use of a Packard beta-counter at an efficiency of 50%. Specific binding was defined as total binding minus nonspecific binding. Specific binding was directly proportional to the protein concentration. Data were analyzed with a Scatchard plot allowing Bμ and Kd calculations.

Competition Experiments
Competition experiments were performed with [3H]-AF-DX384 binding (5 nMol/L) in the presence of increasing concentrations of carbachol, a nonselective muscarinic agonist (ranging from 10-11 to 10-3 mol/L). Buffer, incubation procedures, rapid filtration, and radioactivity retained on filters were realized as described above. Curves were analyzed with a 2-site model according to n Hill. Competition curves were analyzed to calculate IC50 (eg, calculated concentration of carbachol able to inhibit 50% of maximal specific binding) for both high- and low-affinity agonist binding sites.

Evaluation of Adenylyl Cyclase Activity
As described by Salomon et al, 21 adenylyl cyclase activity was evaluated in right atrial membranes from both dog groups. Briefly, 20 to 30 µg of membranes were incubated in a final volume of 100 µL incubation buffer (40 mMol/L Tris-HCl, 100 µMol/L EGTA, 1.5 mMol/L MgCl2, pH 7.4) containing 0.1 µMol/L guanosine 5'-triphosphate, 1 mMol/L cyclic adenosine 3',5'-monophosphate (cAMP), 0.5 mMol/L isobutylmethylxanthine (IBMX), 0.2 mMol/L adenosine 5'-triphosphate (ATP), 5 mMol/L creatine phosphate, 70 IU/mL creatine kinase, 0.2% bovine serum albumin, and 0.5 to 1 µCi [32P]-ATP. Direct and indirect adenylyl cyclase stimulation was achieved with forskolin, a direct adenylyl cyclase agonist (10 µMol/L), or isoproterenol, a nonselective β-adrenoceptor agonist (10 µMol/L), respectively. For the inhibition of adenylyl cyclase activity, membranes were incubated with 5 increasing concentrations of carbachol ranging from 10-8 to 10-3 mol/L in the presence of forskolin (10 µMol/L) or isoproterenol (10 µMol/L).

Incubations were performed for 10 minutes at 30°C, and the reaction was stopped by adding 20 µL of 2.2N HCl and 10 nCi [3H]-cAMP. The incubates were poured off in Dowex columns and eluted with 100 mMol/L ammonium acetate buffer (pH 7.4). The radioactivity contained in the eluate was measured by liquid scintillation using a Packard beta-counter. All results are expressed as picomoles of cAMP formed per milligram of membrane proteins per minute.

Drugs
[3H]-AF-DX 384, [(±)-5,11-dihydro-11-(2 to 2'-[dipropylamino]-methyl]-1-piperidinyl-ethyl) amino] carbonyl-6H-pyrido (2,3-b)1,4-benzodiazepine-6-one] (specific radioactivity 100 to 160 Ci/mmol), [3H]-adenosine 3',5'-cyclic monophosphate (specific radioactivity 25 to 40 Ci/mmol), and [α32P]-adenosine 5'-triphosphate were from New England Nuclear (Boston). Guanosine 5'-triphosphate, adenosine 3',5'-cyclic monophosphate, creatine kinase, and creatine phosphate were purchased from Boehringer-Mannheim. Carbachol and methacholine were provided by Sigma Chemical Co.
A significant increase in systolic BP (161±5 mm Hg) and diastolic BP (92±3 mm Hg) and in HR (96±4 bpm) after 9 weeks of hypercaloric regimen. In the control group, body weight (11.5±0.5 vs 12.0±0.5 kg), systolic BP (148±5 mm Hg) and diastolic BP (82±3 mm Hg), and HR (82±4 bpm) remained unchanged during the entire experimental period.

Circadian Variability of BP and HR
During the baseline period, night values of HR and systolic and diastolic BPs were significantly lower than day values in both control and HFD groups (Figure 1 and Table).

In the control group, differences in HR and BP between day and night were still observed after 9 weeks of normal regimen. In the HFD group, circadian cycles of HR and BP were abolished after 9 weeks of hypercaloric hyperlipidic regimen. Moreover, after 9 weeks of HFD, night and day values of BP and HR were significantly higher than baseline values.

Methylscopolamine Dose-Response Experiments
In both groups, pretreatment with propranolol induced a significant decrease in HR (Figure 2). In the control group, the change in HR was unaffected during the entire experimental period (ΔHR=-23±2 and -25±4 bpm). In the HFD group, the propranolol-induced decrease in HR was significantly increased starting from the third week (ΔHR=-30±1, -30±1, -34±1, -35±1, and -39±2 bpm at baseline period and after 1, 3, 5, 7, and 9 weeks of HFD, respectively).

In controls, methylscopolamine induced a dose-dependent increase in HR (Eₘₐₓ=113±12 bpm; E₅₀=5.0±0.1 μg/kg).

Day and Night Values of HR and Systolic and Diastolic BPs Measured in Control and HFD Groups

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control Group (n=6)</th>
<th>HFD Group (n=6)</th>
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<tr>
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<td>Night</td>
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<td>139±1*</td>
</tr>
<tr>
<td>9</td>
<td>146±3</td>
<td>136±2*</td>
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<tr>
<td></td>
<td>83±1</td>
<td>78±2</td>
</tr>
<tr>
<td>9</td>
<td>81±2</td>
<td>71±2*</td>
</tr>
</tbody>
</table>

During baseline period (week 0) and after 9 weeks of normal and HFD, values of BP and HR were calculated in 2 distinct periods (day from 6 am to 7 pm and night from 11 pm to 6 am) in control and HFD groups, respectively.

Values are expressed as mean±SEM. Single statistical comparisons were performed using paired Student’s t test.

Results

Body Weight, Systolic and Diastolic BPs, and HR
During the baseline period, mean values of HR (78±6 vs 81±3 bpm), systolic BP (143±4 vs 145±4 mm Hg), and diastolic BP (79±5 vs 79±2 mm Hg) were not significantly different in control and HFD groups, respectively. HFD induced a significant body weight increase in dogs (12.0±0.7 vs 14.9±0.6 kg, P<0.005). This gain was associated with a significant increase in systolic BP (161±5 mm Hg) and diastolic BP (92±3 mm Hg) and in HR (96±4 bpm) after 9 weeks of Hypercaloric regimen. In the control group, body weight (11.5±0.5 vs 12.0±0.5 kg), systolic BP (148±5 mm Hg) and diastolic BP (82±3 mm Hg), and HR (82±4 bpm) remained unchanged during the entire experimental period.
agonist. High-affinity receptors represented 29% of the presence of 2 sites with different affinities for muscarinic carbachol resulted in a biphasic curve compatible with the 6

$E_{\text{max}}$ curve was not significantly different from that of control dogs (111 ± 5 bpm; ED$_{50}$=4.8 ± 0.1 μg/kg). In the HFD group, the baseline methyl-scopolamine dose-response curve was not significantly different from that of control dogs (111 ± 5 bpm; ED$_{50}$=4.8 ± 0.1 μg/kg). Methylscopolamine ED$_{50}$ was similar at baseline until the third week of HFD (5.7 ± 0.4, 5.4 ± 0.2) and increased significantly (7.0 ± 0.8, 6.7 ± 0.3, and 7.4 ± 0.4 μg/kg, P<0.05, respectively) from the fifth to the ninth weeks of HFD. During HFD, HR values (before propranolol pretreatment) were positively related to the logarithm of the methylscopolamine ED$_{50}$ (r=0.915 P<10$^{-5}$). HFD failed to induce significant changes in maximal induced tachycardia (E$_{\text{max}}$=102 ± 14, 101 ± 5, 94 ± 7, 88 ± 3, and 95 ± 2 bpm after 1, 3, 5, 7, and 9 weeks, respectively).

**[3H]-AF-DX384 Binding**

In saturation experiments, M$_2$-cholinoceptor $B_{\text{max}}$ measured in the right atrium of dogs submitted to a hyperlipidic hypercaloric diet was significantly lower than in control dogs (27±6 vs 54±6 fmol/mg of membrane protein), with no change in the values of $K_d$ (1.0±0.2 vs 1.2±0.3 nmol/L). In atrium cardiomyocyte membranes from control dogs, competition of [3H]-AF-DX384 binding by increasing concentrations of carbachol resulted in a biphasic curve compatible with the presence of 2 sites with different affinities for muscarinic agonist. High-affinity receptors represented 29±2% (IC$_{50}$=2.0±0.5 nmol/L) and low-affinity receptors 71±3% (IC$_{50}$=1.0±0.3 μmol/L) of the total atrial population of M$_2$-cholinoceptors.

In the HFD group, the percentage of high- and low-affinity receptors (33±2% and 67±3%, respectively) was not significantly different from control values. However, the IC$_{50}$ for both high- and low-affinity agonist binding sites were significantly right-shifted in obese hypertensive dogs (274±40 nmol/L and 61±3.0 μmol/L, respectively; Figure 3). Moreover, M$_2$-cholinoceptor $B_{\text{max}}$ was significantly correlated with both HR values and the logarithm of methylscopolamine ED$_{50}$ (r=−0.761, P=0.0027, and r=0.680, P=0.013, respectively).

**Adenylyl Cyclase Activity**

In control atrial membranes, compared with basal value (58±15 pmol cAMP/min/mg membrane protein), incubation with isoproterenol or forskolin induced a significant increase in adenylyl cyclase activity (126±17 and 148±17 pmol cAMP/min/mg membrane protein, respectively, P<0.05). In atrial membranes from HFD dogs, basal adenylyl cyclase activity as well as the stimulating effects of both isoproterenol and forskolin were not significantly different from control dogs (68±5, 104±5, and 148±11 pmol cAMP/min/mg membrane protein, respectively).

The ability of carbachol to inhibit the activity of adenylyl cyclase stimulated by isoproterenol (Figure 4, top) or forskolin (Figure 4, bottom) was significantly lower in obese hypertensive dogs (IC$_{50}$=47±12 and 172±49 μmol/L, respectively) than in controls (IC$_{50}$=6.4±1.4 and 1±0.3 μmol/L, respectively). The logarithm of methylscopolamine ED$_{50}$ was also correlated with the logarithm of the agonist IC$_{50}$ evaluated after isoproterenol stimulation (r=0.835, P=0.0003).

**Discussion**

Our results show that the decrease in the activity of the effenter parasympathetic limb of the baroreflex can be explained by an impairment in cardiac muscarinic cholinoceptor function in obesity-related hypertension. For the first time, a decreased in number and agonist affinity of atrial M$_2$-cholinoceptors without any functional change in adenylyl cyclase per se is described in obesity-related hypertension.

In this model of the obese hypertensive dog, which also presents sodium and fluid retention, an increase in free fatty acid plasma levels, and insulin resistance without hyperglycemia, the first result to be discussed is the abolition of the circadian cycle of HR and BP. This modification, probably because of a decreased parasympathetic activity, appears to be specifically related to the appearance of obesity-related hypertension. It was not observed in lean and normotensive dogs, in which, as previously reported, we found a 10% reduction in both HR and BP during the night period, which was stable over the experimental period. Circadian rhythm of BP and HR is mainly related to changes in autonomic nervous system activity during the night. In fact, treatment with atropine was shown to abolish the nocturnal decrease of HR in rats, thus indicating the physiological role of the parasym-
pathetic nervous system in this periodic oscillation. In the same way, in patients with autonomic failure with parasympathetic dysfunction, abolition of the circadian rhythm of HR is an early event and is related to dysautonomia. Arterial hypertension during hyperlipidic hypercaloric diet is associated with both diurnal and nocturnal tachycardia caused by parasympathetic tone decrease. Despite the lack of change in cardiac muscarinic receptor changes observed in obesity-related hypertension is associated with a decrease in number and affinity of peripheral M2-cholinceptors.

The determination of adenylyl cyclase activity first gives further insight into the results of the binding studies. The ability of carbachol to inhibit the stimulated adenylyl cyclase activity is reduced in obese hypertensive dogs. The activity of adenylyl cyclase, in basal conditions as well as after isoproterenol or forskolin stimulation, remains similar in obese hypertensive and normotensive dogs; this functional alteration was not due to a change in adenylyl cyclase activity per se. As for in vivo and competition experiments, the observed changes in adenylyl cyclase activity could also be explained by a reduction of atrial M2-cholinceptors number or by a loss in their affinity for agonists.

As in human right atrium, the M2-cholinceptor subtype predominates in canine heart. Through inhibitory G proteins, muscularic receptors inhibit adenylyl cyclase activity and decrease heart rate.

Figure 4. Carbachol inhibition curves of forskolin- and isoproterenol-stimulated adenylyl cyclase activity. Forskolin (top) and isoproterenol (bottom) adenylyl cyclase stimulation were achieved with forskolin (10 μmol/L) or adenylyl cyclase (10 μmol/L), respectively. Adenylyl cyclase activity was evaluated in right atrial membranes from control and HFD dogs. For the inhibition of adenylyl cyclase activity, membranes were incubated with 5 increasing concentrations of carbachol ranging from 10^-6 to 10^-5 mol/L. Results are presented as the percentage of inhibition of the enzyme activity after stimulated by forskolin or isoproterenol. Values are expressed as mean±SEM. Statistical comparisons were performed with ANOVA followed by Dunnett’s post hoc test. A value of P<0.05 was considered significant. *Significant difference between control and HFD dogs.

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Although further studies could be performed to confirm these M₂-cholinergic changes in obese hypertensive humans, our study supports that impaired M₂-cholinergic function is associated with tachycardia and changes in short- and long-term (eg, circadian) BP and HR variabilities in obesity-related hypertension. These abnormalities are well known as risk factors for cardiovascular death related to human arterial hypertension. The data suggest that atrial M₂-cholinergic receptor stimulation is able to downregulate M₂-cholinergic receptors. So, as suggested by propranolol blockade, early sympathetic overactivity rather than arterial hypertension could lead to changes in vagal tone in obesity-related hypertension.

In summary, we found that the decrease in parasympathetic tone induced by HFD in dogs is related to an impaired atrial M₂-cholinergic receptor without changes in adenylyl cyclase activity. The decrease in parasympathetic tone could reduce the capacity of the autonomic nervous system to buffer BP changes. Endogenous catecholamines are able to downregulate M₂-cholinergic receptors. So, as suggested by propranolol blockade, early sympathetic overactivity rather than arterial hypertension could lead to changes in vagal tone in obesity-related hypertension.

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References
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