Age-Dependent Changes of Cardiac Neuronal Noradrenaline Reuptake Transporter (Uptake1) in the Human Heart

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OBJECTIVES
The purpose of this study was to elucidate whether the neuronal noradrenaline reuptake transporter (uptake1) undergoes age-dependent regulation in the human heart.

BACKGROUND
Aging is associated with various alterations in cardiovascular function. We determined uptake1 density (by [3H]-nisoxetine binding to membranes) and activity (by accumulation of [3H]-noradrenaline into tissue slices) in the right atria (RA) of 42 patients (age range 3 months to 76 years) undergoing open-heart surgery without apparent heart failure. Moreover, the effects of 1 μmol/l desipramine on the noradrenaline-induced positive inotropic effect were assessed in the isolated, electrically driven RA trabeculae of these patients.

RESULTS
There was a significant negative correlation between RA uptake1 density and age; moreover, RA uptake1 activity was significantly reduced in elderly patients. Desipramine (1 μmol/l) significantly shifted noradrenaline concentration-response curves to the left; this shift was significantly more pronounced in younger patients than in older patients.

CONCLUSIONS
With increasing age, human myocardial uptake1 activity decreases, possibly because of age-dependent downregulation of uptake1 density. (J Am Coll Cardiol 2002;40:1459–65) © 2002 by the American College of Cardiology Foundation

Aging is associated with reduced responsiveness of many hormone receptors. Numerous studies in animal models of aging, but also in humans, have shown that cardiac beta-adrenoceptor function declines with aging (1–3). The mechanism underlying this age-dependent reduction in cardiac beta-adrenoceptor function is not completely understood. It has repeatedly been shown that plasma noradrenaline levels are higher in elderly than in young people (4–6). Measurements of plasma noradrenaline levels are generally accepted. It has repeatedly been shown that plasma noradrenaline levels are higher in elderly than in young people. (4–6). Measurements of plasma noradrenaline levels are generally taken as an index of sympathetic activity (7); thus, increases in plasma noradrenaline might reflect increases in sympathetic activity with aging. Such an increased sympathetic activity, which has been directly demonstrated by microneurographic recordings from the sympathetic nerves of elderly subjects (8,9), could likely cause prolonged stimulation of cardiac beta-adrenoceptors, which finally leads to desensitization of cardiac beta-adrenoceptor function often observed in the elderly.

However, in the synaptic cleft, action of noradrenaline at the receptors is terminated by reuptake into sympathetic nerve terminals through the neuronal uptake1 transporter (10,11). Thus, an age-dependent reduction in activity of this transporter would also lead to prolonged action of noradrenaline at beta-adrenoceptors and could thus contribute to cardiac beta-adrenoceptor desensitization with aging. In fact, there is indirect evidence that, with aging, neuronal uptake of noradrenaline declines (6,12). However, direct measurements of neuronal uptake1 sites and neuronal uptake1 activity in aging human hearts are missing.

We have recently shown that, in rat cardiac membranes, (3H)-nisoxetine binding and, in rat cardiac tissue slices, assessment of (3H)-noradrenaline accumulation can be used to identify sites that have characteristics of the cardiac neuronal uptake1 transporter (13).

In this study, we used these techniques to assess the density and activity of the neuronal uptake1 transporter in the right atria (RA) obtained from 42 patients (age range 3 months to 76 years) during open-heart surgery without apparent heart failure. Moreover, to get further insight into the functional consequences of possible changes in uptake1 activity, we assessed, in the isolated, electrically driven RA trabeculae of these patients, the effects of the uptake1 blocker desipramine on positive inotropic effects evoked by noradrenaline through beta-adrenoceptor stimulation.

METHODS

Patients. Right atrial appendages were obtained from: 1) 19 patients (9 male and 10 female; age range 3 months to 21 years) with acyanotic congenital heart disease who underwent open-heart surgery because of a ventricular septal defect (n = 8) or atrial septal defect (n = 11). Their parents had given informed, written consent. The study was approved by the local Ethics Committee. None of the children...
Abbreviations and Acronyms
RA = right atrium/atria/atrial
NAT = neuronal noradrenaline transporter
NYHA = New York Heart Association

Contraction studies. Preparation of tissues was performed in oxygenated Tyrode's solution (see previous description) at room temperature to minimize inadequate oxygenation. Right atrial appendages were dissected to yield trabecular strips (4–5 mm in length and ≤1 mm in diameter) without endocardial damage and with fibers running parallel to the length. Preparations were used only if at least two functioning trabecular strips were obtained. Preparations were mounted in a 10-ml organ bath containing carbenogated Tyrode's solution at 37°C. Atrial strips were electrically stimulated by rectangular pulses (5 ms) ~20% above the threshold (mean 8 V; range 3–12) at a frequency of stimulation of 1.0 Hz (Stimulator II, Hugo Sachs Elektronik KG, March-Hugstetten, Germany). The developed tension of the preparation (maintained under a rest tension of 4.9 mN) was recorded via a strain gauge on a Hellige recorder (Hellige GmbH, Freiburg, Germany). Preparations were allowed to equilibrate for at least 1 h in Tyrode's solution; phentolamine (1 μmol/l) was present throughout the experiments to block alpha-adrenoceptors.

Thereafter, preparations were incubated with 1 μmol/l desipramine or vehicle for 30 min, followed by cumulative concentration–response curves for noradrenaline (10⁻⁸ to 10⁻⁴ mol/l) constructed as detailed elsewhere (16). Each muscle preparation was used for one concentration–response curve only to exclude desensitization phenomena.

(³H)-nisoxetine binding. To determine neuronal noradrenaline transporter (NAT) density, crude membranes were isolated as recently described (13), with minor modifications. Briefly, frozen tissue was thawed on ice in ice-cold incubation buffer (mmol/l: Na₂HPO₄ 10, NaCl 120, KCl 5; pH 7.4) containing 0.25 mol/l sucrose. Fatty and connective tissues were carefully removed, and 100 to 150 mg of the remaining tissue was minced with scissors and gradually homogenized with an Ultra-Turrax (Ultra-Turrax T25, Janke & Kunkel IKA Labortechnik, Germany) at 24,000 rpm for 10 s and twice at 17,500 rpm for 20 s with 1-min intervals on ice. The homogenate, brought up to 10 ml with incubation buffer (with 0.25 mol/l sucrose), was centrifuged for 10 min at 1,200 g at 4°C. The supernatant was filtered through four layers of cheesecloth and centrifuged for 20 min at 20,000 g at 4°C. The resulting pellet was suspended in 10 ml of incubation solution (without sucrose), and the protein content was determined according to Bradford (17), using bovine gamma-globulin as a standard. Saturation analysis of (³H)-nisoxetine was performed by incubating 100 μg protein/assay with six concentrations of (³H)-nisoxetine, ranging from 0.3125 to 10 nmol/l in a final volume of 500 μl for 3 h at 4°C. Nonspecific binding of (³H)-nisoxetine was defined as binding in the presence of 1 μmol/l desipramine. Specific binding was defined as total binding minus nonspecific binding and amounted to ~80% at 2 nmol/l (³H)-nisoxetine. All incubations were performed in duplicate.

To assess the ability of the noradrenaline uptake inhibitors desipramine, nisoxetine, and cocaine and the dopamine

Table 1. Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Group A (n = 19)</th>
<th>Group B (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>12 ± 3</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>71 ± 3 (3)</td>
<td>62 ± 5 (12)</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>86 ± 7 (8)</td>
<td>71 ± 6 (16)</td>
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Data are presented as the mean value ± SEM or number of patients (in parentheses).

HR = heart rate; LVEF = left ventricular ejection fraction.

or young adults had acute heart failure or had been treated with sympathomimetic or sympatholytic agents for at least three weeks before the operation. Anesthesiologic premedication and surgery were carried out exactly as recently described (14,15). Right atrial appendages were removed immediately after installation of the cardiopulmonary bypass. 2) Twenty-three adult patients with coronary artery disease (18 male and 5 female; age range 45 to 76 years) undergoing coronary artery bypass graft surgery without apparent heart failure (New York Heart Association [NYHA] functional class 0/I: n = 5; class II: n = 18) after having given informed, written consent. The study was approved by the local Ethics Committee. None of these patients had been treated with sympathomimetic or sympatholytic drugs for at least six weeks before the operation. However, patients had received nitrates (n = 9), calcium antagonists (n = 3), diuretics (n = 6), hydroxymethyl glutaryl coenzyme A reductase inhibitors (n = 16), acetyl salicylic acid (n = 7), and, occasionally, digitalis glycosides (n = 2), alone or in combination. Anesthesiologic premedication and surgery were carried out exactly as recently described (14,15). In all patients, RA appendages were removed during installation of cardiopulmonary bypass.

Immediately after excision, the specimens were either quickly frozen in liquid nitrogen and stored at −80°C (for (³H)-nisoxetine binding) or transferred into carbenogated (95% O₂/5% CO²) Tyrode's solution (mmol/l: NaCl 119.8, KCl 5.4, CaCl₂ 1.8, MgSO₄ 1.05, NaH₂PO₄ 0.42, NaHCO₃ 23.6, glucose 5.05, EDTA 0.05, and ascorbic acid 0.28; at room temperature) and transported to the laboratories. Experiments for positive inotropic effects in RA trabeculae were started usually within 15 to 30 min after excision; experiments for (³H)-noradrenaline accumulation into RA tissue slices were started within 1 to 2 h after excision. Patients were classified into two age groups: group A included children (mean age 12 ± 3 years; range 3 months to 21 years); group B included elderly patients (mean age 60 ± 3 years; range 45 to 76 years). The clinical characteristics of the patients are given in Table 1.

Table 1. Clinical Characteristics of the Patients
transporter antagonist GBR 12909 to inhibit specific \(^{(3)}\text{H}\)-nisoxetine binding, membranes (100 \(\mu\)g protein/assay) were incubated with 2 nmol/l \(^{(3)}\text{H}\)-nisoxetine in the presence of 10 different concentrations of the competing agent for 3 h at 4\(^\circ\)C, and specific binding was determined as described earlier.

\(^{(3)}\text{H}\)-noradrenaline uptake. Activity. Activity of NAT was assessed as recently described (13), with minor modifications. Briefly, RA appendages transported in Tyrode’s solution were allowed to equilibrate in modified and carboxenated Krebs-Henseleit solution (mmol/l: NaCl 118, KCl 4.7, CaCl\(_2\) 2.5, MgCl\(_2\) 0.54, NaHCO\(_3\) 25, NaH\(_2\)PO\(_4\) 1, glucose 11, EDTA 0.094, ascorbic acid 1.14, nialamide 0.067) for at least 30 min at room temperature. After equilibration, fatty and connective tissues were carefully removed, and the remaining RA appendage tissue was chopped into 250 \(\mu\)m slices with a McIlwain tissue chopper (Bachhofer, Reutlingen, Germany). Slices were resuspended in fresh Krebs-Henseleit solution and allowed to equilibrate for further 15 min at 37\(^\circ\)C. Activity of NAT was measured by incubating 10 mg tissue slices/assay at 37\(^\circ\)C for 15 min in carboxenated Krebs-Henseleit solution containing 1.56, 3.125, 6.25, 12.5, and 25 nmol/l \(\text{D,L-}(7-\text{H})\)-noradrenaline hydrochloride in the presence of 40 \(\mu\)mol/l corticosterone (specific uptake\(_2\) blocker) in a final volume of 500 \(\mu\)l. Nonspecific accumulation of radioactivity was determined by parallel incubation at 4\(^\circ\)C. Specific uptake was defined as total uptake (37\(^\circ\)C) minus nonspecific uptake (4\(^\circ\)C). All incubations were performed in duplicate.

**Statistics.** Experimental data given in the text, figures, and table are expressed as the mean value \(\pm\) SEM of the number of experiments. The equilibrium dissociation constant (\(K_{D}\)) and the maximal number of binding sites (\(B_{\text{max}}\)) for \(^{(3)}\text{H}\)-nisoxetine binding were calculated by nonlinear regression analysis (hyperbolic function: \(y = B_{\text{max}} \times x/(K_D + x)\)), using the iterative curve-fitting Prism program (Graph-Pad Software, San Diego, California). One-site competition curves (\(y = \text{bottom} + [\text{top} - \text{bottom}]/[1 + 10^{(x - \log EC_{50})}]\), with \(x = \log [\text{antagonist concentration}]\) and \(y = \text{\(^{(3)}\text{H}\)-noradrenaline binding}\) were analyzed using the iterative curve-fitting program Prism from which the individual IC\(_{50}\) values (concentration of competitor to inhibit specific \(^{(3)}\text{H}\)-nisoxetine binding by 50\%) were obtained. Experimental data of contraction studies were fitted and analyzed by computer-supported iterative nonlinear regression analysis (sigmoidal concentration-response curve: \(y = \text{bottom} + [\text{top} - \text{bottom}]/[1 + 10^{[x_0 \log EC_{50} - x]}]\), with \(x = \log [\text{agonist concentration}]\) and \(y = \text{response}\), using the Prism program. Linear regression analysis of data was performed by the least-squares method (model: \(^{(3)}\text{H}\)-nisoxetine binding sites = \(a \times \text{age} + b\)). The significance of differences was estimated by using the nonpaired two-tailed Student \(t\) test or, to evaluate possible gender differences, by repeated measures analysis of variance followed by the \(t\) test with the Bonferroni correction for multiple comparisons (age, \(B_{\text{max}}\), and gender: \(n = 18\)). All statistical calculations were performed using the Prism program. A \(p\) value \(<0.05\) was considered significant.

**Drugs used.** \(\text{D,L-}(7-\text{H[N]}\)-noradrenaline hydrochloride (specific activity: 13.5 Ci/mmole) and \((N\)-methyl-\(^{(3)}\text{H}\)-)nisoxetine (specific activity: 80 Ci/mmole) were purchased from NEN Life Science Products, Inc. (Boston, Massachusetts); desipramine hydrochloride from RBI (Natick, Massachusetts); and corticosterone, (-)-noradrenaline bitartrate, and nialamide from Sigma (Deisenhofen, Germany). All other chemicals were of the purest grade commercially available.

**RESULTS**

\(^{(3)}\text{H}\)-nisoxetine binding. \(^{(3)}\text{H}\)-nisoxetine binding (0.3125–10 nmol/l) to RA membranes was saturable and had a high affinity (\(K_{D}\) values \(\sim1\) nmol/l) (Fig. 1A). Uptake inhibitors inhibited \(^{(3)}\text{H}\)-nisoxetine binding with the following order of potency: desipramine \(\geq\) nisoxetine \(\geq\) cocaine \(\geq\) GBR 12909 (Fig. 1B), which is typical of noradrenaline uptake\(_1\) (13).

However, the maximal number of binding sites in group A (7 children [5 males and 2 females]; mean age 14 \pm 3 years [range 4 to 21]) was significantly higher (163 \pm 4.5 fmol/mg protein) than that in group B (11 elderly patients [8 males and 3 females]; mean age 60 \pm 3 years [range 45 to 76]) (105 \pm 8.2 fmol/mg protein; \(p < 0.001\)) (Fig. 1A). Moreover, for all 18 patients, the RA NAT density was significantly negatively correlated with age (\(p < 0.0001\)) (Fig. 2). The \(K_{D}\) values for \(^{(3)}\text{H}\)-nisoxetine, however, were not significantly different between group A (1,091 \pm 101 pmol/l) and group B (724 \pm 214 pmol/l). Repeated measures analysis of variance of these data revealed no statistically significant influence of gender.

\(^{(3)}\text{H}\)-noradrenaline uptake. Next, we determined NAT activity by assessment of \(^{(3)}\text{H}\)-noradrenaline (1.56–25 nmol/l) (see Methods) accumulation in RA slices. As shown in Figure 3, \(^{(3)}\text{H}\)-noradrenaline accumulation in RA slices was directly related to the \(^{(3)}\text{H}\)-noradrenaline concentration present in the media. Moreover, at each \(^{(3)}\text{H}\)-noradrenaline concentration, the NAT activity was significantly higher in group A (children) than in group B (elderly patients) (Fig. 3). However, because of a limited amount of tissue, we could only determine the \(^{(3)}\text{H}\)-noradrenaline accumulation at all five \(^{(3)}\text{H}\)-noradrenaline concentrations in four of the seven children, whereas in the remaining three children, the \(^{(3)}\text{H}\)-noradrenaline accumulation was determined only at the 25 nmol/l \(^{(3)}\text{H}\)-noradrenaline concentration. Thus, in addition, we calculated the data obtained at 25 nmol/l \(^{(3)}\text{H}\)-noradrenaline in all seven children (group A) and in five elderly patients (group B). This analysis revealed that NAT activity was significantly higher (208 \pm 13 pmol noradrenaline/mg tissue per 15 min) in seven children of group A (mean age 4 \pm 3 years [range 3 months to 6 years]; \(n = 7\)) than in five elderly patients of group B (mean age 59
potency of noradrenaline (pD₂ value = negative logarithm of the molar concentration of noradrenaline causing half-maximal effects) was 5.8 ± 0.03 in group A and 6.0 ± 0.06 in group B. Desipramine at 1 μmol/l increased the basal force of contraction (see legend to Fig. 4) and shifted the concentration-response curves of noradrenaline to the left (Fig. 4). However, this shift was significantly larger in children (ΔpD₂ value: 1.2 ± 0.05) than in elderly patients (ΔpD₂ value: 0.8 ± 0.06; p < 0.001).

DISCUSSION

With aging, plasma noradrenaline increases 10% to 15% per decade due to enhanced spillover of noradrenaline into the circulation (12,18). Such increased plasma noradrenaline levels could be the result of increased sympathetic activity or decreased plasma clearance of noradrenaline through uptake₁ (12,19). Increased sympathetic activity with aging has been demonstrated by direct microneurographic recordings from sympathetic nerves to skeletal muscle (8,9). However, indirect evidence from noradrenaline-spillover studies indicate that an age-dependent reduction in NAT activity might contribute to the increase in plasma noradrenaline with aging (1,12,20).

Uptake₁ is reduced in the aging human heart. We have recently shown that, in rat ventricular crude membranes, (³H)-nisoxetine binding was inhibited by uptake₁ inhibitors with the following order of potency: desipramine > nisoxetine >> cocaine ≥ GBR 12909 (13). In this study, uptake₁ inhibitors inhibited (³H)-nisoxetine binding to human RA membranes with the same order of potency (Fig. 1B). Moreover, the same order of potency was also found for inhibition of (³H)-noradrenaline accumulation (measure of NAT activity) in rat ventricular slices (13). Thus, assessment of (³H)-noradrenaline accumulation in cardiac slices...
and (³H)-niosoxetine binding to cardiac membranes can be used to determine cardiac NAT activity and density.

In the present study, we determined, by these techniques directly, human cardiac NAT density and activity in RA appendages from patients with a wide range of age. We found that NAT density was significantly lower in elderly patients (mean age 60 ± 3 years) than in children (mean age 14 ± 3 years). This age-dependent decrease in NAT density was accompanied by a similar loss in NAT activity; in elderly patients, NAT activity was 50% lower than that in children. Such an age-dependent loss in NAT activity could explain why the myocardial noradrenaline content is reduced in aging hearts (21,22).

The mechanism underlying decreases in cardiac NAT density and activity with aging is not known at present; however, it should be considered that in the human myocardium, there is a progressive age-dependent loss of myocytes (23) and sympathetic nerves (24), which might likely contribute to a reduction in transporter density with aging.

**Beta-adrenoceptors are desensitized in the aging human heart.** It is well known that with aging, cardiac beta-adrenoceptor function declines (1,2,3,25). We have previously shown that in the RA of patients of different ages (7 days to 83 years), beta-adrenoceptor-mediated adenylyl cyclase activation significantly decreased with age; beta-adrenoceptor density, however, was only marginally altered. Moreover, in these RA, the potency of isoprenaline to increase the force of contraction significantly decreased with age (14). Similarly, decreased beta-adrenoceptor-mediated adenylyl cyclase activation and inotropic potency of isoprenaline were also found in ventricular preparations of aging human hearts (26–28). However, this was accompanied by decreased beta-adrenoceptor density (27). Thus, human cardiac beta-adrenoceptors are also desensitized with increasing age (3,25).

**Functional consequence of reduced uptake** in the aging human heart. In the present study, however, the potency of noradrenaline to induce positive inotropic effects in isolated RA was very similar in young and elderly patients. However, isoprenaline is not a substrate for NAT (29,30), although noradrenaline is. Therefore, it is very likely that, due to reduced NAT activity (as seen in the present study), noradrenaline is not as sufficiently inactivated in elderly patients as in young patients. This results in higher noradrenaline concentrations at beta-adrenoceptors, thus masking the reduced beta-adrenoceptor responsiveness usually seen in elderly patients. If this hypothesis holds true, one should expect that in the presence of uptake blockers, concentration-response curves for positive inotropic effects of noradrenaline should be shifted to the left and more...
pronounced in young than in elderly patients. This was, indeed, the case (Fig. 4): in the RA from young patients, desipramine shifted the noradrenaline concentration-response curve to the left by 1.2 log units, whereas in elderly patients, this shift was only 0.8 log units (Fig. 4).

The present result of reduced NAT activity in elderly patients might also explain the differences in the effects of age on heart rate responses to isoprenaline and adrenaline infusion, as recently described by White and Leenen (31,32): heart rate increases were nearly identical in young (age 30 years) and elderly volunteers (age 60 years); ganglionic blockade with trimethaphan unmasked decreased chronotropic responses to isoprenaline in elderly patients, but did not affect adrenaline-induced heart rate increases. As mentioned earlier, isoprenaline is not a substrate for uptake1, although adrenaline is. Thus, it is very likely that, due to reduced NAT activity, during adrenaline infusion, adrenaline concentrations at beta-adrenoceptors are higher in elderly volunteers than in young volunteers, thereby compensating for the age-dependent decrease in chronotropic response to beta-adrenoceptor stimulation.

**Study limitations.** In this study, we observed age-dependent differences in children versus elderly patients. Children were quite young (mean age 12 years), and elderly patients were not completely healthy but had a mild cardiac diagnosis. Thus, the changes observed in this study may not be entirely due to aging but may have occurred during maturation or may be, at least partly, due to the beginning of mild heart failure (in all subsets of patients studied, NYHA functional class was <II). We cannot exclude these possibilities, and it will be rather impossible to obtain tissue samples from normal human hearts from patients with a more appropriate age distribution. However, we recently found in patients with a similar age distribution, significant correlations between age and RA adenylyl cyclase activation (14), as well as age and RA muscarinic receptors (15). Thus, we are quite sure that, in this study, changes in RA uptake1 activity are predominately due to aging.

**Conclusions.** The density of the RA NAT is decreased in patients 60 years old, as compared with young patients, and this is accompanied by decreased NAT activity. The pathophysiologic consequence of such decreased NAT activity could be increased noradrenaline concentrations in the synaptic cleft at beta-adrenoceptors, and this might contribute to desensitization of cardiac beta-adrenoceptors often observed in aging.

Finally, the present results add further to the growing list of similarities between aging and failing human hearts: in both settings, plasma catecholamines are increased; the myocardial catecholamine content is decreased; and the responses to stimulation of all receptors that involve intracellular cyclic adenosine monophosphate increases are diminished (3,25,33). In addition, not only in failing human hearts (34,35), but also in aging human hearts (as in the present study), NAT density and activity are diminished.

**Figure 4.** Effects of 1 μmol/l desipramine (DMI) on the positive inotropic effect of noradrenaline (in the presence of 1 μmol/l phentolamine) in right atrial (RA) trabecular strips obtained from 5 children in group A (A, left panel) (3 males and 2 females; mean age 9 ± 4 years) and from 7 elderly patients in group B (B, right panel) (5 males and 2 females; mean age 60 ± 3 years) (New York Heart Association functional class 1.71 ± 0.18). Ordinates: positive inotropic effect in percent maximal response; abscissa: molar concentrations of noradrenaline. The basal force of contraction was 2.5 ± 0.4 mN (without DMI) and 5.0 ± 0.8 mN (with DMI) in group A and 3.6 ± 0.6 mN (without DMI) and 4.2 ± 1.3 mN (with DMI) in group B. The maximal force of contraction was 7.2 ± 0.6 mN (without DMI) and 6.7 ± 1.0 mN (with DMI) in group A and 8.7 ± 0.7 mN (without DMI) and 6.1 ± 1.3 mN (with DMI) in group B. Data are presented as the mean value ± SEM.
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