ALTERATION OF NOREPINEPHRINE RELEASE FROM [3H]-NOREPINEPHRINE PRELOADED BASILAR ARTERY BY NAPHTHALENESULFONAMIDES

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Accepted March 24, 1982

Abstract—The effects of W-7, W-5, No. 233, and chlorpromazine on sympathetic nerve transmitter efflux were compared in superfused canine basilar arterial preparations preloaded with [3H]-norepinephrine. In vitro experiments suggest that these agents are selective calmodulin antagonists. The electrical transmural stimulation-induced efflux of tritium was reduced by W-7 and W-5, although they were unexpectedly equipotent since W-5 is a chloride-deficient derivative of W-7 and has a lower affinity for calmodulin than does W-7. The median inhibitory concentration (IC50) of W-7 for stimulation-induced efflux was $3.4 \times 10^{-6}$ M. The addition of No. 233 at relatively high concentrations ($3 \times 10^{-5}$ M and $5 \times 10^{-5}$ M) caused a reduction in stimulation-induced efflux. Chlorpromazine produced a dual effect on the efflux: enhancement at low concentrations (below $1 \times 10^{-6}$ M) and reduction at high concentrations. The IC50 values of No. 233 and chlorpromazine were $3.5 \times 10^{-5}$ M and $2.5 \times 10^{-5}$ M, respectively. The additions of these four agents also caused a significant elevation in the spontaneous basal efflux of tritium from the preparations. The concentrations of the agents that elevated the spontaneous efflux to the level of half the stimulation-induced efflux were closely fitted to the IC50 values for stimulation-induced efflux. This finding indicates that the elevation in spontaneous efflux is directly proportional to the reduction in electrical stimulation-induced efflux. From these findings, it is concluded that naphthalenesulfonamides including W-7 have a direct effect on sympathetic nerve terminals which is independent of the effect on calmodulin.

Calmodulin antagonists such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine, pirenylamine and N²-dansyl-L-arginine-4-t-butylpiperidine amide (No. 233) are recognized to serve as useful pharmacological tools for elucidating the biological significance of Ca²⁺, calmodulin-mediated reactions (1–9). In our previous report using isolated rabbit aortic strips, we demonstrated that among these calmodulin antagonists, W-7 is apparently the most specific for calmodulin (10). However, synthetic compounds usually have multiple pharmacological effects such as have been demonstrated for chlorpromazine and other psychotropic drugs (10–12). Chlorpromazine at low concentrations exhibits a specific antagonism against norepinephrine (NE), serotonin (5-HT), and

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histamine (10). Prenylamine is a specific blockade of transmembrane influx of Ca\(^{2+}\) (13, 14). No. 233 selectively antagonized the 5-HT- and KCl-induced contractions in isolated rabbit aortic strips (10). Relatively high concentrations of these calmodulin antagonists reportedly exhibited inhibitory effects on calmodulin (1–10). In the case of W-7, this antagonist has at least two kinds of pharmacological actions on isolated vascular strips: one is calmodulin-related vascular relaxation and another is calmodulin-independent contraction (15). The vascular contractile effect of W-7 is due to the release of endogenous NE from sympathetic nerve terminals (15). N- (6-aminohexyl)-1-naphthalenesulfonamide (W-5) is a chloride-deficient derivative of W-7 and has a lower affinity for calmodulin than does W-7. W-5 produced a significant release of NE from rabbit aortic strips, but did not produce aortic relaxation which is clearly demonstrated in the response of W-7 (15). From this evidence, it is likely that the effect of chloride-deficient naphthalenesulfonamide derivative, or the release of NE, is not the result of an effect on calmodulin.

To further clarify the calmodulin-independent effects of naphthalenesulfonamides, we determined the effects of W-7, W-5, No. 233, and chlorpromazine on \[^3H\]-NE release from canine basilar arterial preparations.

**MATERIALS AND METHODS**

**Preparation of arterial strip:** Mongrel dogs of either sex weighing 8–13 kg were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and sacrificed by bleeding from the common carotid arteries. The basilar artery from the brain was quickly excised. The artery was helically cut at an angle of approximately 45° to the longitudinal axis resulting in strips of 1 mm in width and 15 mm in length according to the method of Lewis and Koessler (16) or Furchgott and Bhadrakom (17). The strips were preincubated with 1 x 10^{-7} M \[^3H\]-norepinephrine (\[^3H\]-NE, specific activity 24.7 Ci/mmol: New England Nuclear) in 1 ml of modified Krebs' bicarbonate solution equilibrated with 95% O\(_2\) and 5% CO\(_2\). The composition of the modified Krebs' solution was as follows (in millimolar concentrations): NaCl, 115.0; KCl, 4.7; CaCl\(_2\) \cdot 2H\(_2\)O, 2.5; MgCl\(_2\) \cdot 6H\(_2\)O, 1.2; NaHCO\(_3\), 25.0; KH\(_2\)PO\(_4\), 1.2; and dextrose, 10.0. Ascorbic acid at a concentration of 100 \(\mu\)g/ml was always added to the solution. The pH of the solution was 7.5. After incubation for 60 min with \[^3H\]-NE at 37 °C, the strip was suspended by threads between two parallel platinum wire electrodes (30 mm length, 1.5 mm apart) fixed vertically on opposite sides of the strip, as previously described by Su and Bevan (18). The upper end of the strip was connected to the lever of a force-displacement transducer (TB-612T, Nihon Kohden Kogyo, Co., Tokyo, Japan). An initial resting tension of 1 g was applied to the arterial strips. The basilar arterial strips were superfused with prewarmed (37°C) and oxygenated modified Krebs' solution at a constant flow rate of 1 ml/min.

Rabbit (2.3–2.7 kg) basilar artery and thoracic aorta were also examined. Helical strips of these arteries were preincubated with \[^3H\]-NE and fixed vertically between the electrodes.

Preparations were allowed to equilibrate for 90 min under superfusion conditions before the onset of stimulation protocols.

**Stimulation parameters:** Transmural electrical stimulation was delivered across the platinum wire electrodes via an electronic stimulator (SEN-3201, Nihon Kohden Kogyo, Co., Tokyo, Japan). Stimulation parameters (0.3 msec duration, 20 V across the electrodes) were chosen to achieve supramaximal nerve stimulation. Strips were stimulated for 10 sec at a frequency of
Efflux of $[^3\text{H}]$-NE: The spontaneous and stimulation-induced efflux of tritium from the strips was determined by assaying 1.0 ml-aliquots of the superfusate collected in vials which were changed at 1 min intervals. Ten milliliters of Aqueous Counting Scintillant (Amersham/Searle Co., Des Plaines, IL) were subsequently added to the aliquots in the vials. Tritium activity in the collected superfusate was counted to a 1% error in a Beckman LS-9000 liquid scintillation counter with automatic external standardization to determine efficiency. Stimulation-induced efflux is expressed as disintegrations per minute (dpm) and was determined by subtracting the spontaneous efflux, determined from the mean of the two 1-min samples taken immediately prior to stimulation, from the stimulation-induced efflux.

After transmural stimulation, collection was continued until tritium levels returned to the spontaneous levels. For example, with canine basilar artery where stimulation was administered for 10 sec, the efflux in the second poststimulation collection was almost always at spontaneous levels; and in the third poststimulation collection, this was always the case.

Data are routinely expressed as the ratio of stimulation-induced efflux in a second period of stimulation in the presence of the desired test drug to that obtained in the first period of stimulation in the absence of drug (19–22). This procedure nulls out any differences between preparations in the absolute amounts of tritium released since the effect of a drug is determined by a same strip comparison of stimulation-induced efflux first in the absence and then in the presence of drug. A matching group of controls serves to correct and null out (normalize) any changes in efflux due to time alone. Results shown in the text, Table, and Figures were expressed as the mean value±S.E. For statistical evaluation, data were analyzed by the Student’s t-test, paired t-test or analysis of variance (23). Statistical significance was assumed when P<0.05.

Drugs and chemicals: /-$[^3\text{H}]$-norepinephrine (specific activity 24.7 Ci/mmol) was purchased from New England Nuclear, Boston, Massachusetts. Other substances employed included N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5), N²-dansyl-L-arginine-4-t-butylpiperidine amide (No. 233), chlorpromazine hydrochloride (Smith Kline and French Laboratories), tetrodotoxin (Sankyo Co., Tokyo), and phentolamine mesylate (Regitine mesylate, Ciba Pharmaceutical Co., Summit, NJ). All drugs were added to the superfusing modified Krebs’ solution.

RESULTS

Efflux of $[^3\text{H}]$-NE induced by transmural stimulation in canine basilar artery: Canine basilar arterial strips usually responded to electrical transmural stimulation for 10 sec at 20 Hz with outputs of tritium which were substantially elevated above spontaneous levels (Fig. 1). The mean stimulation-induced tritium efflux determined in 12 control strips from 12 dogs was 869±171 dpm/mg wet weight in the first trial and only slightly and insignificantly less, 801±154 dpm/mg wet weight, when an identical period of stimulation was repeated after a 30-min interval. Three sequential trials with an interval of 30 min in the basilar artery are shown in Fig. 1A. The ratio of second period vs. first period stimulation values for the 12 control strips was 0.93±0.04.

The spontaneous tritium efflux from the basilar artery is also shown in Fig. 1A. The spontaneous efflux in the 12 control strips before the first stimulation period was 755±69 dpm/mg wet weight, and it was
Fig. 1. Tritium efflux from canine basilar artery by electrical transmural stimulation. The preparations were preloaded with [3H]-NE for 60 min and then superfused with modified Krebs' solution at 37°C. (A) Three sequential trials of stimulation with an interval of 30 min. (B) Effects of W-7 on stimulation-induced tritium efflux. W-7 at a concentration of 2.5 x 10^-6 M was added 10 min before the second stimulation (TS2). The stimulation was then repeated in the presence of W-7. TS: transmural stimulation for 10 sec with an intensity of 20 V at a frequency of 20 Hz. Abscissa: time after incubation with [3H]-NE.

Fig. 2. Effects of W-7 on the tritium efflux induced by transmural stimulation in canine basilar arterial, rabbit basilar arterial, and rabbit aortic strips. The value of stimulation-induced efflux in the presence of W-7 was expressed as the percentage of the 12 untreated control strips (i.e., 2nd/1st ratio as percentage of the control). Superfusion of various concentration of W-7 was started 10 min before the second stimulation. Data points are the mean±S.E. of 6 to 8 determinations.

significantly less, 636±59 dpm/mg wet weight (P<0.05), when measured before the second stimulation period. The ratio of second period vs. first period values for the 12 control strips was 0.840±0.004.

Effects of two naphthalenesulfonamides on stimulation-induced efflux: To determine the effects of W-7 on transmitter efflux, preparations were first stimulated at 20 Hz for 10 sec and then exposed to this agent for 10 min before the second stimulation. The stimulation was then repeated as in the control with strips in the presence of W-7. The addition of W-7 in a concentration of 2.5 x 10^-6 M caused a slight increase in the spontaneous tritium efflux and concomitantly reduced the stimulation-induced tritium efflux (Fig. 1B). The preparation was then superfused with a normal solution not containing W-7 and stimulated at a third period with an interval of 30 min, the effect of W-7 was almost disappeared (Fig. 1B).

Concentration-dependent inhibition by W-7 of the stimulation-induced tritium efflux from canine basilar arterial strips is shown in Fig. 2. To assess the general applicability of the inhibition to vascular tissue the effect of W-7 was examined in strips of rabbit basilar artery and thoracic aorta. Rabbit basilar arterial preparations were stimulated at 20 Hz for 10 sec in the same way as previously described for the canine basilar artery, and rabbit aortic preparations were stimulated using the same parameters except with an intensity of 50 V. The results obtained are shown in Fig. 2. W-7 in concentrations ranging from 2.5 x 10^-6 M to 2 x 10^-5 M also caused a concentration-dependent inhibition of stimulation-induced tritium efflux in these two preparations. Median inhibitory concen-
trations (IC50) of W-7 for the stimulation-induced efflux in these arteries were $3.2 \times 10^{-6}$ M (canine basilar artery), $3.7 \times 10^{-6}$ M (rabbit basilar artery), and $1.3 \times 10^{-5}$ M (rabbit aorta).

The addition of W-5 in concentrations ranging from $5 \times 10^{-6}$ M to $3 \times 10^{-5}$ M also caused a concentration-dependent reduction in stimulation-induced tritium efflux from canine basilar artery (Table 1). The IC50 value of W-5 for the stimulation-induced efflux was $6.2 \times 10^{-6}$ M. W-5 produced an increase in the spontaneous tritium efflux (Table 1).

Effect of other calmodulin antagonists on stimulation-induced efflux: Effects of No. 233 and chlorpromazine were also studied in canine basilar arteries (Table 1). The addition of No. 233 in concentrations ranging from $1 \times 10^{-5}$ M to $5 \times 10^{-5}$ M caused a concentration-dependent reduction in stimulation-induced efflux (Table 1). Chlorpromazine reduced the efflux at the highest concentration ($1 \times 10^{-4}$ M), but not at the lower concentrations used. Effects of various concentrations of chlorpromazine on stimulation-induced tritium efflux are shown in Fig. 3. Chlorpromazine at a concentration of $1 \times 10^{-6}$ M caused a 2-fold increase in stimulation-induced efflux. Enhancement by

| Agents$^a$ | Conc. ($\times 10^{-6}$ M) | N | Stim.-induced Efflux Ratio: 2nd/1st$^b$ | Spontaneous Efflux Ratio: 2nd/1st$^b$ |
|------------|-----------------------------|---|--------------------------------------|--------------------------------------|
| W-7        | 2.5                         | 8 | 0.57±0.04***                        | 1.07±0.03***                        |
|            | 5                           | 8 | 0.52±0.05***                        | 1.48±0.07***                        |
|            | 10                          | 8 | 0.15±0.05***                        | 2.79±0.19***                        |
|            | 30                          | 6 | 0.02±0.01***                        | 7.82±1.24***                        |
| W-5        | 5                           | 6 | 0.51±0.04***                        | 1.07±0.06***                        |
|            | 10                          | 6 | 0.37±0.04***                        | 1.60±0.13***                        |
|            | 30                          | 6 | 0.08±0.03***                        | 3.86±0.18***                        |
| No. 233    | 10                          | 6 | 0.84±0.13                           | 0.84±0.01                           |
|            | 30                          | 6 | 0.53±0.08***                        | 1.18±0.06***                        |
|            | 50                          | 6 | 0.02±0.01***                        | 2.90±0.24***                        |
| Chlorpromazine | 1                           | 6 | 1.92±0.39**                         | 0.89±0.03*                          |
|            | 10                          | 6 | 1.28±0.19*                          | 1.08±0.06***                        |
|            | 30                          | 4 | 0.90±0.10                           | 1.23±0.16***                        |
|            | 100                         | 3 | 0.07±0.03***                        | 2.79±0.28***                        |

$^a$ Agents were administered for 10 min before the second period of stimulation as described in the text.

$^b$ The ratios of transmitter efflux in the first and second periods of electrical transmural stimulation in the absence and presence of the agents. The ratio of second period vs. first period stimulation values for 12 untreated control strips to which all other groups were compared was 0.93±0.04.

$^c$ The spontaneous efflux values were determined in two 1-min samples taken immediately before the onset of the first and second periods of stimulation. The ratio of second period vs. first values for the 12 untreated control strips to which all other groups were compared was 0.840±0.004.

N indicates the number of preparations used. Data are expressed as the mean±S.E. *P<0.05, **P<0.01, ***P<0.001 as compared to ratio for the control strips.
Fig. 3. Effects of various concentrations of chlorpromazine on transmural stimulation-induced tritium efflux in canine basilar artery. On the left hand scale, the value of stimulation-induced efflux in the presence of chlorpromazine was expressed as the percentage of the 2nd/1st ratio as percentage of the control and on the right hand scale as the percentage of inhibition of presynaptic α-adrenergic receptors (see "Discussion"). Data points are the mean±S.E. of 4 to 6 determinations, except for the point at 1×10^{-4} M chlorpromazine which is the mean of 3 determinations. Chlorpromazine was added 10 min before the second stimulation, and the stimulation was repeated in the presence of chlorpromazine.

Chlorpromazine of transmural stimulation-induced tritium efflux can be explained through the presynaptic α-adrenergic receptor hypothesis (24–27) since chlorpromazine is an α-adrenergic receptor blocking agent. At 1×10^{-4} M, chlorpromazine caused an almost complete inhibition of stimulation-induced efflux (Table 1, Fig. 3). Fifty percent inhibition of the presynaptic α-adrenergic receptor was exhibited at a chlorpromazine concentration of 2.5×10^{-5} M. The IC50 values of No. 233 and chlorpromazine were significantly higher than that of W-7.

Elevation in spontaneous efflux by naphthalenesulfonamides and other calmodulin antagonists: When the arterial strips was exposed to 2.5×10^{-6} M W-7, there was observed a slight but a significant increase in spontaneous tritium efflux (Fig. 1B); thereupon, transmural stimulation was administered. A strip exposed to 5×10^{-6} M W-7 also produced an increase in spontaneous efflux. The effect of each of the calmodulin antagonists on spontaneous tritium efflux at the concentrations employed is shown in Table 1. All three calmodulin antagonists, namely, W-7, No. 233, and chlorpromazine modestly increased the spontaneous efflux at almost all the concentrations employed. Furthermore, W-5 also produced an elevation in the spontaneous efflux (Table 1). As clearly shown in the table, a similar statistical significance existed between the reduction in stimulation-induced tritium efflux and the elevation in spontaneous efflux.

In order to determine the possibility that the elevation in spontaneous efflux affects the stimulation-induced efflux, the following analysis was carried out: The increased amount of spontaneous efflux at the stimulation period in the presence of 2.5×10^{-6} M W-7 corresponded to 17.8% of the stimulation-induced efflux (horizontal line of Fig. 4B). This value of "expected percent inhibition" was calculated from the following
equation:

\[
\text{"expected percent inhibition"} = \left( \frac{SE_{W-7} - SE_2}{TSE_2} \right) \times 100 (\%) \quad (1)
\]

SE_{W-7} and SE_2 refer to the spontaneous efflux at the second period of stimulation in the presence and the absence of W-7, respectively; whereas, TSE_2 was expressed as transmural stimulation-induced efflux at the second period in the absence of W-7. In this equation, SE_2 and TSE_2 can be calculated as SE_1 \times 0.84 and TSE_1 \times 0.93 (SE_1, spontaneous efflux at first period of stimulation and TSE_1, transmural stimulation-induced efflux at first period), hence SE_2 and TSE_2 were changed to 84% of SE_1 and 93% of TSE_1, respectively. Therefore, equation (1) can also be written as follows:

\[
\text{"expected percent inhibition"} = \left( \frac{SE_{W-7} - (SE_1 \times 0.84)}{TSE_1 \times 0.93} \right) \times 100 (\%) \quad (2)
\]

Figure 4A shows the control stimulation-induced efflux determined at the second period in the absence of W-7 (TSE_2). The “expected percent inhibition” value of 5 \times 10^{-6} M W-7 was calculated as 65.5% of the stimulation-induced efflux, and this value is shown in Fig. 4C. From the “expected percent inhibition” values shown in Fig. 4B and Fig. 4C the concentration of W-7 which caused an elevation in spontaneous efflux to 50%-level of stimulation-induced efflux was then determined. A value of 4.0 \times 10^{-6} M is the result of such a determination: it shows that the “expected IC50 value” determined from the “expected percent inhibition” is almost identical with the IC50 value for stimulation-induced efflux as previously described. The “expected IC50 values” determined from equation (2) and the method shown in Fig. 4 were 4.0 \times 10^{-6} M (W-7), 7.0 \times 10^{-6} M (W-5), 3.2 \times 10^{-5} M (No. 233) and 3.1 \times 10^{-5} M (chlorpromazine). The correlation between the reduction in stimulation-induced efflux and the elevation in spontaneous efflux is shown in Fig. 5. The figure indicates that the elevation in spontaneous efflux is directly proportional to the reduction in transmural stimulation-induced efflux. NCM103, another derivative of W-7, which possesses a carboxyl substituent in the alkyl group of the structure of W-7 and a lower affinity for calmodulin, neither produced an elevation in spontaneous efflux nor a reduction in the stimulation-induced efflux up to the concentration of 1 \times 10^{-4} M (data not shown).

Effects of tetrodotoxin and phentolamine: Confirmation that the stimulation-induced efflux of tritium in canine basilar arteries, rabbit basilar arteries, and rabbit aorta was due to nerve activation was done by a 10-min exposure of superfused tissues to tetrodotoxin (1 \times 10^{-7} M), which selectively paralyzes
nerve function (28). In the presence of tetrodotoxin, the stimulation-induced efflux of tritium with 200 pulses at 20 Hz was reduced to 0.4, 0.3, and 1.2% of matching control values in 5 canine basilar arterial, 4 rabbit basilar arterial, and 5 rabbit aortic strips respectively. These data indicate that the increased tritium efflux was due to stimulation of intramural nerves. Phentolamine, an α-adrenergic receptor blocking agent caused a significant increase in stimulation-induced efflux. The ratio of second period vs. first period stimulation in the presence of $1 \times 10^{-6}$ M phentolamine was $2.06 \pm 0.25$ ($n=7$, $P<0.001$).

**DISCUSSION**

In the present study, putative calmodulin antagonists, namely, W-7, No. 233, and chlorpromazine had antagonistic effects on transmural electrical stimulation-induced $[3H]$-NE efflux from canine basilar arteries. However, the effect would not be predicted to be due to an interaction with calmodulin for a number of reasons. When the effect of W-7 was compared with that of W-5, a chloride-deficient derivative of W-7 which has a much lower affinity for calmodulin than does W-7 (15), W-5 exhibited a similar antagonistic effect on $[3H]$-NE efflux. Moreover, No. 233 and chlorpromazine that are both potent antagonists against calmodulin produced a weaker inhibition of $[3H]$-NE efflux than does W-7 (4). All these findings taken together suggest that the inhibition by these compounds of stimulation-induced $[3H]$-NE efflux is a calmodulin-independent action.

Interestingly, these four compounds produced an increase in spontaneous $[3H]$-NE efflux from canine basilar arteries. A good correlation between the inhibition of stimulation-induced $[3H]$-NE efflux and the increase in spontaneous $[3H]$-NE efflux was obtained as shown in Fig. 5. It is likely that the two naphthalenesulfonamides, No. 233, and chlorpromazine cause a release of endogenous NE and thereby suppresses the stimulation-induced release of NE from sympathetic nerve terminals. This represents the adrenergic presynaptic receptor mechanism. The adrenergic presynaptic receptor mechanism is a good hypothesis as an acceptable explanation for the effects of agonists and antagonists on sympathetic nerve transmitter release (24–27). Exogenously added NE and other sympathomimetics inhibit the stimulation-induced release of NE and phenoxybenzamine, and other α-receptor antagonists block this effect and by themselves increase the release of transmitter. This mechanism was also demonstrated in isolated canine basilar arteries (29). It has already been reported that chlorpromazine increased the release of NE (22, 30, 31), and this effect also occurred in the absence of $Ca^{2+}$ (30, 31). As is clearly shown in Fig. 3, chlorpromazine produced dual effects on the release of NE: enhancement at low concentrations and inhibition at high concentrations. In an attempt to visualize the release modulating capacity of presynaptic receptors and the extent of activation of these receptors by released endogenous NE, we have drawn the right-hand scale in Fig. 3 according to the method of Wemer et al. (32). This scale is based on the assumption that the maximal reduction of NE release through the endogenous NE secretion by chlorpromazine indicates 100% activation of the presynaptic receptors, and the maximal increase by the compound has been considered to indicate a complete blockade of the presynaptic receptors. Therefore, the 50%-inhibition of presynaptic receptors was attained at a concentration of $2.5 \times 10^{-5}$ M of chlorpromazine by using the right-hand scale.

Calmodulin-independent release of NE from canine basilar artery by W-7 is considered to be a nonspecific effect of naph-
thalenesulfonamides since W-5 also exhibited a similar effect on this preparation. W-5 seems to serve as a so-called control agent of the calmodulin antagonist W-7, and combination study of this pair of compounds should give information on the pharmacological relevance of the calmodulin interaction with antagonists.

Acknowledgments: This work was supported in part by Grant-In-Aid for Scientific Research (B) 448114 from the Ministry of Education, Science and Culture, Japan and by a grant from the Japanese Atherosclerosis Foundation.

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