Differential Inotropic Effects of 4-Aminopyridine and Tetraethylammonium on Rat Diaphragm and Limb Muscles

Erik van Lunteren

Departments of Medicine and Neurosciences, Case Western Reserve University and Cleveland VA Medical Center, Cleveland, USA

Abstract

K^+ channels regulate action potential duration and thereby force of skeletal muscles. Diaphragm differs from limb muscles in the dependence of contraction on extracellular Ca^{++}. We hypothesized that diaphragm also differs from limb muscles in the regulation of contraction by membranous K^+ conductances. This was tested *in vitro* by assessing changes in isometric twitch force and kinetics in response to K^+ channel blockers, and comparing responses of rat diaphragm with that of two limb muscles, the extensor digitorum longus (which contains mainly fast-twitch fibers) and the soleus (which contains mainly slow-twitch fibers). 4-Aminopyridine (0.3 mM) increased twitch force of diaphragm by 71 ± 7%, which was significantly more than that of the extensor digitorum longus (28 ± 11%, $P < 0.005$) and the soleus (22 ± 3%, $P < 0.005$). In contrast, tetraethylammonium (10 mM) increased twitch force of the diaphragm by 9 ± 1%, which furthermore was smaller than that of the extensor digitorum longus (41 ± 2%, $P < 0.001$) and soleus (53 ± 3%, $P < 0.001$) muscles. There was also a differential pattern among muscles in percent prolongation of isometric contraction time, which paralleled that of twitch force augmentation. Charybdotoxin (10 nM), apamin (100 nM) and glibenclamide (100 μM) did not alter muscle isometric twitch force or kinetics of any muscle. Thus the pattern of diaphragm muscle responses to the K^+ channel blockers 4-aminopyridine and tetraethylammonium differs from that of limb muscles. This can not be attributed to differential blockade among muscles of ATP-sensitive or Ca^{++}-activated K^+ channels, but could be explained if diaphragm contains different delayed rectifier K^+ channel subtypes from those found in limb muscle.

Key words: diaphragm, limb muscle, rat, contraction, 4-aminopyridine, tetraethylammonium.

Basic Appl. Myol. 9 (2): 53-59, 1999

In skeletal muscle, the duration of the action potential influences muscle contractile performance by regulating the mechanically effective contractile period. Longer action potential durations enhance Ca^{++} release from the sarcoplasmic reticulum and augment muscle contractile force [13, 20]. The speed with which the membrane repolarizes and hence the duration of the action potential is in turn determined to a large extent by the rapidity and degree to which K^+ conductance increases. Altering K^+ conductance can profoundly affect contractility. For example, in mouse diaphragm 4-aminopyridine (4-AP), tetraethylammonium (TEA) and uranyl nitrate increase twitch force by up to 55% singly and by ~200% when given in combination [16], in rat diaphragm 4-AP, 3,4-diaminopyridine (DAP) and TEA increase twitch force by ~40-60% [8, 25, 26], and in rat diaphragm increases in force induced by 4-AP and DAP persist over several minutes of repetitive stimulation [25, 28].

Regulation of ionic conductances and transport varies among skeletal muscles. In some cases, diaphragm differs from limb muscle, e.g. for degree of dependence of contraction on extracellular Ca^{++} [4, 29]. The present study tested the hypothesis that there is heterogeneity among skeletal muscles in the degree to which various K^+ channel blockers modulate isometric twitch force. Furthermore, differential responses among muscles to pharmacologic interventions are determined both by their fiber type composition (slow versus fast fibers) and by their functional role (diaphragm versus limb muscle).

Methods

Studies were performed *in* vitro on muscle from adult (200-250 g) Sprague-Dawley rats. All experiments were performed in accordance with the animal care and welfare guidelines of the National Institutes of Health (U.S.A.). The animals were anesthetized with intraperitoneal urethane (1-1.5 g/kg initial dose, with supplemental doses given as needed). The diaphragm, extensor digitorum longus and soleus muscles were removed surgically. In the rat, the diaphragm is comprised of large proportions of both slow and fast fibers (eg. 58% fast and 42% slow fibers) [27], the extensor digitorum longus muscle is comprised almost exclusively of fast fibers (eg. 97-98% fast fibers) [2, 3], and the soleus muscle is comprised predominantly of slow fibers (eg. 77-96% slow fibers reported in various studies) [1-3]. The muscles were placed in oxygenated physiologic solution, and small strips (diameter 1-1.5 mm) were made keeping the cartilaginous and tendinous origins and insertions intact. The muscles were mounted vertically in a double jacketed bath (temperature 37°C) containing physiological solution comprised as follows (in mM): NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 15, glucose 11, with the pH adjusted to 7.3-7.4 while being aerated with 95% O_2 - 5% CO_2. The muscles were stimulated electrically (pulse width 1 ms, supramaximal voltage) at optimal length via platinum electrodes, and force was measured with a high-sensitivity isometric transducer (Kent Scientific Corporation/Radnotti Glass Technology, Monrovia, CA). Addition of curare (0.025 mM) to the bath in preliminary studies did not alter twitch force, confirming that the muscles were activated directly.

Muscle strips underwent isometric twitch stimulation at a frequency of 0.1 Hz. Following a three minute baseline period, aliquots of drugs dissolved in physiological solution (pH adjusted) were added to the bath. Twitch force continued to be monitored thereafter during continuous 0.1 Hz stimulation. The incubation time chosen for each agent was that required for twitch force to reach its maximum increase (or at least 10 minutes if no increase was apparent). DMSO, in which some drugs were initially dissolved, had no effect on twitch force in the concentrations used. Muscle strips in which force varied by more than 5% during the baseline period were rejected from further analysis. All reagents and drugs were obtained from Sigma (St. Louis, MO), except for TEA chloride and charybdotoxin which were obtained from Research Biochemicals Inc. (Natick, MA).

Muscle force records were digitized, collected on line (Axotape, Axon Instruments, Foster City, CA), and stored on the hard drive of a computer. On-screen measurements of force were made with manually controlled cursors. Isometric twitch force was measured in grams, and changes in force in response to K^+ channel blockers were expressed relative to the last five twitches during the baseline period. Isometric twitch kinetics were quantified by measurements of contraction time (the time between the onset of force production and the achievement of peak force) and the half-relaxation time (the time required for peak force to decay by 50%). Statistical analysis was performed with ANOVA followed by the Newman-Keuls test for comparisons of multiple groups, and by linear regression for relating force with isometric twitch kinetics. A P value of < 0.05 (two-tailed) was considered to indicate statistical significance.

Results

4-aminopyridine and tetraethylammonium

The aminopyridines (4-AP, DAP) and TEA each block many but not all types of K^+ channels in skeletal muscle. An example of the effects of 4-AP on diaphragm and soleus muscle twitch force is depicted in Figure 1A. In this example, twitch force increased gradually over the course of approximately eight minutes, and the magnitude of force increase was considerably greater for the diaphragm than for the soleus muscle. The increase in force was accompanied by a prolongation of contraction time, as depicted for the diaphragm in Figure 1B.

In response to 4-AP (0.3 mM), isometric twitch force of all three muscles increased, with the relative increase of the diaphragm being significantly greater than that of either the extensor digitorum longus muscle or the soleus muscle (Figure 2). In contrast, with TEA (10 mM) the relative increase in twitch force was greatest for the soleus muscle, intermediate for the extensor digitorum longus muscle, and least for the diaphragm. Twitch force of muscle strips in which no drug was added remained relatively stable, declining by a few percent over the same time period (Table 1).

The isometric contraction and half-relaxation times were prolonged to variable extents by 4-AP and TEA (Table 2). Prolongation of contraction time only accounted for part of the force increase, as the rate of force increase was augmented during the latter portion of the twitch (see example for 4-AP in Figure 1B). Isometric twitch kinetics did not change over time in the absence of added drug (data not shown). The percent prolongation of contraction time in response to 4-AP was significantly greater for the diaphragm than the soleus and extensor digitorum longus muscles (Figure 3). Conversely, with TEA the relative prolongation of contraction time was significantly less for the diaphragm than the soleus and extensor digitorum longus muscles. Differential changes among muscles in half-relaxation times were noted with TEA but not 4-AP (Figure 3).

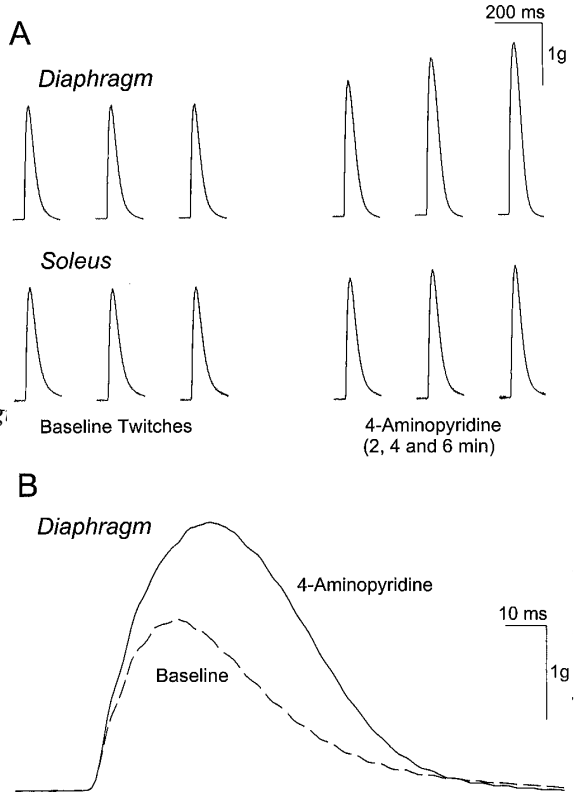

Figure 1. A: Example of changes in diaphragm and soleus muscle twitch force in response to 4-aminopyridine (0.3 mM). Twitches in the presence of 4-aminopyridine were recorded two, four and six minutes after drug addition. B: Time-expanded record of diaphragm twitch force at the time of maximum force increase in response to 4-aminopyridine (approximately eight minutes after drug addition) is superimposed on force during the baseline (control) period.

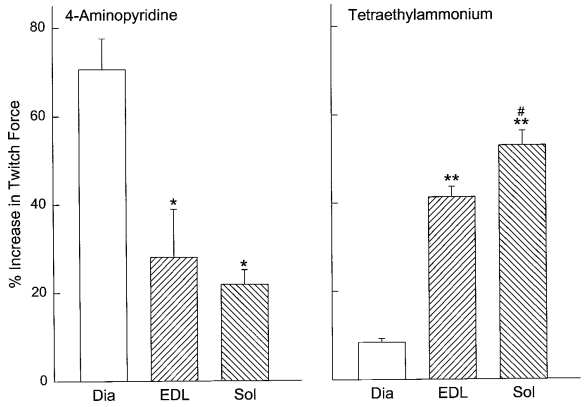

*Figure 2. Maximum increases (mean ± SE) in twitch force of the diaphragm (Dia), extensor digitorum longus (EDL) and soleus (Sol) muscles in response to 4-aminopyridine (0.3 mM) and tetraethylammonium (10 mM). Significant differences between the diaphragm and limb muscles are indicated: ** $P < 0.001$, * $P < 0.005$. Significant differences between soleus and extensor digitorum are also indicated: # $P < 0.01$.*

4-AP and TEA differ in their profiles as blockers of various types of K^+ channels. Blocking delayed rectifier K^+ channels should prolong action potential, and hence would be expected to increase force and prolong the duration of active tension. In contrast, blocking inward rectifier channels should depolarize membrane potential and possibly reduce force, but without affecting the duration of contraction. Mean changes in twitch force were therefore compared with changes in contraction and half-relaxation times in response to 4-AP and TEA (Figure 4). The relative changes in twitch force correlated significantly with the relative changes in contraction time, both when data from the two K^+ channel blockers were pooled ($r = 0.744$, $P < 0.001$) and when each agent was considered separately (see Figure 4). Furthermore, this relationship was similar for the two K^+ channel blockers. On the other hand, changes in twitch force did not correlate with changes in half-relaxation time, either when pooled data were considered ($r = 0.156$, P = not significant) or each K^+ channel blocker was considered separately (Figure 4).

Barium chloride

Barium blocks inward rectifier channels, but may also affect other types of K^+ channels (DiFransesco et al., 1991; Quayle et al., 1988; Standen et al., 1978). Barium chloride (5 mM) increased force of diaphragm and extensor digitorum longus muscles to a greater extent than that of soleus muscle (Figure 5). A similar but non-significant pattern was noted for the prolongation of contraction time, whereas prolongation of half-relaxation time did not differ among muscles (Figure 5).

Table 1. Maximum percent increases in twitch force in response to saline (no drug), charybdotoxin, apamin and glibenclamide.

	Diaphragm	Extensor Digitorum Longus	Soleus
No Drug	-0.1 ± 0.5	1.8 ± 3.3	-0.1 ± 0.4
Sample Size	5	5	6
Charybdotoxin (10 nM)	0 ± 0.1	2.0 ± 2.0	3.7 ± 3.8
Sample Size	5	5	5
Apamin (100 nM)	0.8 ± 0.8	-1.3 ± 1.2	0.1 ± 2.1
Sample Size	6	6	5
Glibenclamide (100 µM)	0 ± 0.3	-0.5 ± 1.2	0.1 ± 0.5
Sample Size	5	5	5

Values are means ± SE. Negative values indicate that force never increased after drug addition.

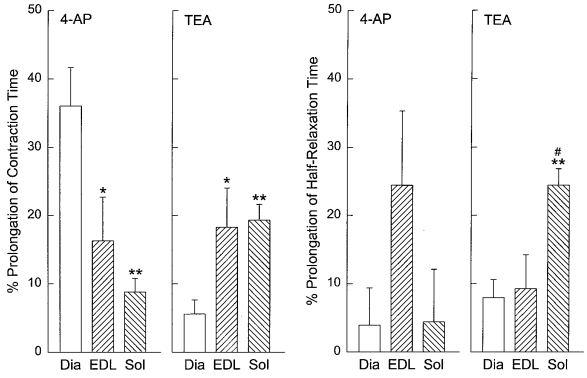

*Figure 3. Changes (mean ± SE) in isometric contraction and half-relaxation times of the diaphragm (Dia), extensor digitorum longus (EDL) and soleus (Sol) muscles in response to 4-aminopyridine (4-AP, 0.3 mM) and tetraethylammonium (TEA, 10 mM). Values are for the same twitches as Figure 2. Significant differences between the diaphragm and limb muscles are indicated: ** P < 0.01, * P < 0.025. Significant differences between soleus and extensor digitorum are also indicated: # P < 0.01.*

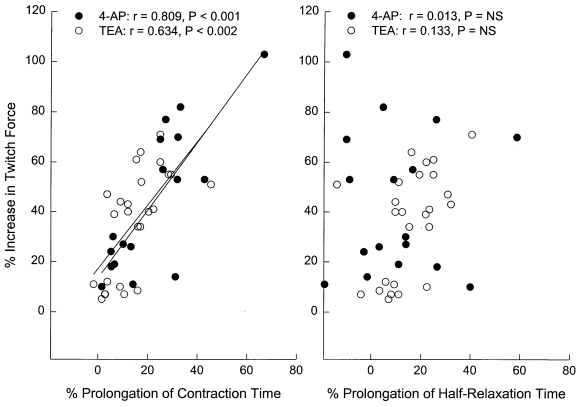

Figure 4. Relationship between increases in peak twitch force and prolongations of isometric twitch kinetics for the diaphragm, soleus and extensor digitorum longus muscles in response to 4-aminopyridine and tetraethylammonium. Data are pooled from all three muscles for each K^+ channel blocker; each point indicates data from one muscle strip. Linear regressions are indicated separately for each K^+ channel blocker, which were significant for that between prolongation of contraction time and increase in force, but not for that between prolongation of half-relaxation time and increase in force.

Charybdotoxin, apamin, and glibenclamide

Charybdotoxin and apamin block high and low-conductance Ca^{++}-activated K^+ channels, respectively, whereas glibenclamide blocks ATP-sensitive K^+ channels (Blatz & Magleby, 1986; Brinkmeier et al., 1991; Comtois et al., 1994; Cook, 1988; Garcia et al., 1991; Light et al., 1994; Quayle et al., 1988). Charybdotoxin (10 nM), apamin (100 nM) and glibenclamide (100 µM) did not alter twitch force of the diaphragm, soleus or extensor digitorum longus muscles (Table 1). Furthermore, these K^+ channel blockers did not change the isometric contraction or half-relaxation times of any muscle

Table 2. Effects of 4-aminopyridine and tetraethylammonium on isometric contraction and half-relaxation times of the diaphragm, extensor digitorum longus and soleus muscles.

	Contraction Time (ms)			Half-Relaxation Time (ms)		
	Diaphragm	Extensor Digitorum Longus	Soleus	Diaphragm	Extensor Digitorum Longus	Soleus
Pre-Drug	21.1 ± 1.1	12.7 ± 0.7	30.2 ± 2.2	22.9 ± 1.5	10.4 ± 0.9	34.1 ± 2.5
4-Aminopyridine	28.4 ± 0.9	14.4 ± 0.7	33.0 ± 2.8	24.0 ± 2.2	12.6 ± 0.7	35.6 ± 3.6
P Value	0.00011	0.054	0.019	0.411	0.075	0.571
Sample Size	7	5	5	7	5	5
Pre-Drug	21.1 ± 0.4	11.3 ± 0.2	26.8 ± 1.1	21.1 ± 1.3	8.7 ± 0.6	28.9 ± 1.3
Tetraethylammonium	22.3 ± 0.6	13.3 ± 0.6	31.8 ± 1.1	22.8 ± 1.5	9.3 ± 0.2	35.8 ± 1.3
P Value	0.030	0.023	0.0000021	0.017	0.222	0.00000022
Sample Size	8	6	11	8	6	11

Values are mean ± SE, and are for the end of the baseline period and at the time of the maximum increase in twitch force.

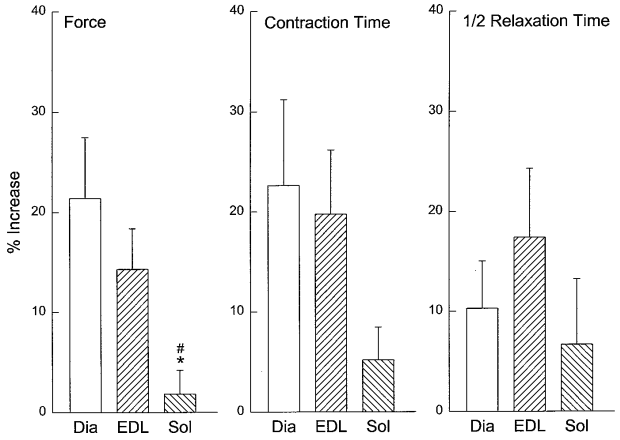

Figure 5. Increases (mean ± SE) in isometric twitch force, contraction time and half-relaxation time of the diaphragm (Dia), extensor digitorum longus (EDL) and soleus (Sol) muscles in response to barium chloride (5 mM). Significant differences between the diaphragm and limb muscles are indicated: $P < 0.01$. Significant differences between soleus and extensor digitorum are also indicated: # $P < 0.025$.

(data not shown).

Discussion

The major finding of the present study was that the diaphragm, soleus and extensor digitorum longus muscles differed in the magnitude of their inotropic re
sponses to the K^+ channel blockers, 4-AP and TEA. Furthermore, fiber type composition did not appear to account for the pattern of differential responses among muscles to K^+ channel blockers. That is, had fiber type been the major determinant, then responses of the diaphragm (which contains both fast- and slow-twitch fibers) to 4-AP and TEA should have been intermediate between those of the extensor digitorum longus muscle (which contains mainly fast-twitch fibers) and the soleus muscle (which contains predominantly slow-twitch fibers). However, this pattern of segregation was not found for either degree of twitch force augmentation or contraction time prolongation; rather, responses of the two limb muscles resembled each other more than that of the diaphragm.

A great diversity of K^+ channels are found in skeletal muscle based on studies of cultured muscle cells and intact muscle fibers. K^+ channels can be classified by electrophysiological or molecular criteria. By electrophysiologically-defined criteria, there are at least two delayed rectifier [7, 10, 11, 17, 22], two inward rectifier [12, 18, 32], two ATP-sensitive [15, 21, 23], and two Ca^{++}-activated [6, 19, 24] K^+ channels. There is even greater diversity among skeletal muscle K^+ channels when defined molecularly, especially for voltage-gated K^+ channels. In rodent skeletal muscle Kv1.1, Kv1.4, Kv1.5, Kv2.1, Kv3.1 and Kv3.4 are present at all ages, Kv1.2, Kv1.6 and Kv4.1 are present only prior to adulthood, and Kv1.3, Kv3.2 and Kv3.3 do not appear to be present [14, 30, 31].

The aminopyridines (4-AP, DAP) and TEA each block many but not all types of K^+ channels in skeletal muscle. Although there is some differential sensitivity among K^+ channels to these agents, the degree is insufficient to allow one to distinguish among the different classes of K^+ channels based on pattern of response to these agents. Glibenclamide blocks skeletal muscle ATP-sensitive K^+ channels, but does not block skeletal muscle voltage-gated or Ca^{++}-activated K^+ channels [15]. Charybdotoxin blocks skeletal muscle high conductance Ca^{++}-activated K^+ channels [24], and in other tissues has been found to also block voltage-gated K^+ channels. Apamin blocks all skeletal muscle low conductance Ca^{++}-activated K^+ channels, but it does not block skeletal muscle high conductance Ca^{++}-activated K^+ channels [6]. The absence of an inotropic response to glibenclamide, charybdotoxin and apamin probably argues against roles for either ATP-sensitive or Ca^{++}-activated K^+ channels in determining the differential responses among muscles to 4-AP and TEA.

Blocking inward rectifier K^+ channels would be expected to depolarize muscle fibers and reduce muscle force. One possible explanation for the present findings is that the muscle types varied in the degree to which 4-AP and TEA blocked inward rectifier K^+ channels, but not in the degree to which these agents blocked delayed rectifier K^+ channels. Neither agent blocks inward rectifier K^+ channels very well. Furthermore, if this were the case, one might expect a difference between 4-AP and TEA in the relationship between the degree of force increase and the prolongation of contraction time (force increases presumably being affected by both delayed and inward rectifier K^+ channels, and alterations of contraction time presumably being affected to a greater extent by delayed than inward rectifier K^+ channels). However, this relationship was almost identical for 4-AP and TEA (Figure 4), arguing against the involvement of inward rectifier channels in determining heterogeneous responses of diaphragm and limb muscle to these K^+ channel blockers. On the other hand, barium caused a smaller degree of force increase for a given prolongation in contraction time than did 4-AP, which may reflect its greater effectiveness as a blocker of inward rectifier K^+ channels.

A possible mechanism accounting for the varied responses among muscles to 4-AP and TEA is if the various subtypes of delayed rectifier K^+ channels found in skeletal muscle have differential sensitivity to various K^+ channel blockers, and furthermore that they are distributed differentially among muscles. More specifically,

the present findings could be explained if channels particularly sensitive to 4-AP are most abundant in diaphragm, and channels particularly sensitive to TEA are most abundant in limb muscle. There is some evidence in support of differential sensitivity among delayed rectifier K^+ channel subtypes to blockers. For example, in Schwann cells α-dendrotoxin blocks only one of three physiologically-defined delayed rectifiers whereas 4-aminopyridine blocks two of three delayed rectifiers [5]. Furthermore, among cloned voltage gated K^+ channels (derived from non-skeletal muscle tissue) Kv1.1, Kv1.2, Kv1.6 and Kv3.4 are much more susceptible to block by α-dendrotoxin than are Kv1.3, Kv1.4, Kv1.5 and Kv3.1 [9]. It is not known whether various delayed rectifier K^+ channels are indeed distributed heterogeneously among skeletal muscles, but such a differential distribution combined with a differential sensitivity to K^+ channel blockers could explain the present findings.

In conclusion, the present study found heterogeneity among skeletal muscles in their inotropic responses to the K^+ channel blockers 4-AP and TEA, with that of the diaphragm differing from that of the limb muscles. The exact type(s) of K^+ channels accounting for these differential effects can not be ascertained from the present study, but the absent inotropic responses of all muscles to glibenclamide, charybdotoxin and apamin argue strongly against ATP-sensitive or Ca^{++}-activated K^+ channels playing roles in the present findings. Further studies are needed to determine whether the various subtypes of voltage gated K^+ channels are distributed heterogeneously among muscles, and whether these subtypes are differentially sensitive to 4-AP and TEA, in order to better understand the mechanism accounting for the present findings.

Acknowledgements

This study was supported in part by the VA Medical Research Service, and NIH grants HL-42215 and AG-10418.

Address correspondence to:

Erik van Lunteren, M.D., Pulmonary Section 111J(W), Cleveland VA Medical Center, 10701 East Boulevard, Cleveland OH 44106, USA, phone 216 791 3800, ext 4616, fax 216 231 3420, Email exv4@po.cwru.edu.

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