Enhanced Nitric Oxide Concentrations and Expression of Nitric Oxide Synthase in Acupuncture Points/Meridians

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ABSTRACT

Objectives: The objectives of this study were to examine the distributions of nitric oxide (NO) in the skin points (acupoints)/meridian regions and determine whether neuronal nitric oxide synthase (nNOS) protein levels were associated with NO concentrations in the areas.

Design: Low skin resistance points (LSRP) on the skin surface in response to electrical stimuli were performed in anesthetized adult rats. The skin together with subcutaneous tissue was isolated in meridian regions from PC 2 to 6, BL 36 to 57, CV 3 to 22, and GV 2 to 14. Control skin tissues were obtained in the areas close to related meridians without containing LSRP. Concentrations of nitrite (NO$_{2}^{-}$), nitrate (NO$_{3}^{-}$), and total NO$_{2}^{-}$ plus NO$_{3}^{-}$ (NO$_{x}^{-}$) were quantified in the skin tissues, micropunches of brain nuclei, and blood vessels in a blinded fashion. Western blots were also conducted using polyclonal anti-nNOS and anti-endothelial nitric oxide synthase (eNOS) antibody in the skin tissues.

Results: NO$_{x}^{-}$ and NO$_{3}^{-}$ concentrations were higher (45 ± 8% and 43 ± 7% in the CV, 47 ± 7% and 51 ± 9% in the BL, and 47 ± 8% and 45 ± 6% in the PC) than those in control regions (p < 0.05, n = 6). NO$_{x}^{-}$ concentrations are 2- to 3-fold greater in skin tissues than those in brain regions and blood vessels (p < 0.05, n = 6–8). nNOS protein levels were consistently increased in the skin regions of BL, PC, and GV meridians compared with their controls (p < 0.05, n = 5–7) but endothelial NO synthase expression was not changed.

Conclusion: This is the first evidence showing that NO contents and nNOS expression are consistently higher in the skin acupoints/meridians associated with low electric resistance. The results suggest that enhanced NO in the acupoints/meridians is generated from multiple resources including neuronal NOergic system, and NO might be associated with acupoint/meridian functions including low electric resistance.

INTRODUCTION

Acupuncture meridian theory (channels and collaterals, jingluo) is an essential pathway system described in traditional Chinese medicine for thousands of years. This system is the central theory of many unconventional medical systems, which deals with physiological regulation and pathological changes of the human body (Qian, 1986; Tang 1987). Radioactive tracer research and biophysical studies have shown that the meridian system may exist in humans and animals (Tiberiu et al., 1981; Zhu and Hao, 1989). However, the chemicals and mechanisms of the meridians and their function are not clear by modern sciences.
Several reports in both humans and animals have shown that acupuncture points (acupoints) possess the characteristics of low electrical resistance (LER), and high electric conductance (Ahu, 1981; Chiou et al., 1998; Fraden, 1979; Luciani, 1978; Reichmanis et al., 1976). Recent studies have demonstrated that LER is present not only in the acupoints, but also over the entire lines (about 1.0 mm in width) of the meridians which are described in traditional charts (Ahu, 1981; Zhu and Hao, 1989). It is well documented that skin electrical resistance depends upon the activity of the sympathetic nervous systems and stimulation of sympathetic pathways lowers the skin resistance levels (Korr et al., 1958; Smith et al., 1988). The concentrations of substance P were higher in the skin and the muscle layer of acupoints than in control points, and the highest amount of substance P was found in the skin area (Chan et al., 1998). Morphological studies have identified that the hair follicles and nervous components are enhanced in the meridians/acupoints, which represent areas of potentially high neuronal activity (Luciani, 1978; Wang et al., 1987).

Recent studies have shown that nitric oxide (NO) is perhaps one of the most important messenger molecules, much like a neurotransmitter with a widespread signaling mechanism and function (Bredt and Snyder, 1992; Ma et al., 1995; Moncada and Higgs, 1991). NO stimulates norepinephrine (NE) release from central and peripheral nervous system, which increases sympathetic nerve activity (Ma and Long, 1991a, 1991b; Lonart et al., 1992; Satoh et al., 1996). Neuronal NO synthase (nNOS) expression is exhibited in the skin tissue (Dippel et al., 1994), and NO concentrations in human skin can be continuously monitored by using dermal microdialysis in vivo (Clough et al., 1998a, 1998b). The chemical lability of NO in cells and tissues has been attributed to a rapid oxidation to both nitrite (NO$\textsubscript{2}^-$) and nitrate (NO$\textsubscript{3}^-$) (Ignarro, 1990, 1993). Our recent results are consistent with others that show that measurements of these two stable metabolites (NO$\textsubscript{2}^-$ and NO$\textsubscript{3}^-$) are very adequate indicators of the concentration of NO in the tissue (Ignarro, 1990, 1993; Ma et al., 1999).

The purpose of the present study was to quantify NO$\textsubscript{2}^-$, NO$\textsubscript{3}^-$, and total NO$\textsubscript{2}^-$ plus NO$\textsubscript{3}^-$ (NO$_x^-$) concentrations in the brain regions, blood vessels, and skin tissues with or without containing acupoints/meridians in rats. Locations of acupoints/meridians were detected by measurements of low skin resistance points (LSRP) corresponding to the acupoints of animals and human described in the Chinese classical topography. Quantification of protein levels of nNOS and endothelial nitric oxide synthase (eNOS) in the skin tissues was also conducted using Western blots.

METHODS

Animal, measurements of skin electrical resistance of acupoints, and tissue preparation

All experiments were performed using adult (5–6 months) male Sprague–Dawley rats. The protocol was approved by the Harbor-UCLA Animal Use Committee, and was in accord with AAALAC and NIH guidelines. The animals were maintained on a 12-hour light–dark cycle in temperature and humidity controlled rooms. Food and water were available ad libitum.

The low skin resistance points (LSRP) were performed in Sprague–Dawley rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.). LSRP on the skin surface along the CV, BL, PC, and GV meridians were tested in each rat and the meridian lines were marked following previously described methods (Chiou et al., 1998; Fraden, 1979; Luciani, 1978; Zhu and Hao, 1989). A research assistant throughout the experiment applied the electrode to the animals with as uniform pressure as possible. The room temperature was maintained at 26–27°C. Rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) were shaved and electrical stimuli was applied using an Acupuncture Meridian Locator, WQ6F30 (Dong Hua Electronic Instrument Factory, Beijing, China) on the skin regions. This instrument has been used to locate exactly all the 12 meridians of 1 mm in width in coinidence with the ancient acupuncture meridian charts (Zhu and Hao, 1989). Pulse current (30–40 Hz) was applied to the skin surface using a stainless steel electrode 1 mm in diameter. A single stimulus consisted of 4–5 applications within a 2– to 3 sec period on each point.
The conductance threshold was expressed in current (μA), which is the same stimuli applied to the acupoint, and the nearest 0.1 μA was determined. LSRP were detected by the stimuli applied to the points which causes more than 50% increase in current value. The occurrence of LSRP was repeated by three measurements and averaged for each point.

LSRP on the skin surface along Tianquan (PC 2) to Neiguan (PC 6) in the Pericardium meridian of Hand-JueYin (PC), Chengfu (BL 36) to Chengshan (BL 57) in the Bladder meridian of Foot-TaiYang (BL), Zhongji (CV 3) to Tiantu (CV 22) in the Ren meridian (CV), and Yaoshu (GV 2) to Dazhui (GV 14) in the DU meridian (GV) were tested in 14 rats. These regions were chosen in the experiments following criteria: (1) acupoints/meridians in the region can be easily identified on the body surface; (2) with enough distance away from other meridians for obtaining control tissues; and (3) represent acupoints/meridians on arm, leg, and trunk. Locations of acupoints/meridians were detected by measurements of LSRP corresponding to the acupoints of animals and human described in the Chinese classical topography (Ahu, 1981; Zhu and Hao, 1989). The meridian lines were marked by connecting LSRP, and 2- to 3-mm width of meridian regions containing LSRP were identified (Zhu and Hao, 1989). The animals were sacrificed with sodium pentobarbital (150 mg/kg, i.p.). The skin together with subcutaneous tissue (around 2- to 3-mm width and 3–4 mm in depth) was isolated in meridian regions from PC 2 to PC 6, BL 36 to BL 57, CV 3 to CV 22, and GV 2 to GV 14. Control skin tissue was obtained in the areas close to a related meridian line without containing LSRP (Chan et al., 1998). The brains, gracilis muscle, superior mesenteric artery, aorta, coronary artery, and pulmonary vessels were also assessed. The samples were quickly removed and initially frozen on dry ice, and then stored at −70°C for measurements of NO metabolites (8 rats) and immunoblot analysis (6 rats), respectively.

**Determination of NO$_2^-$, NO$_3^-$, and NO$_x^-$ concentrations**

Concentrations of NO$_2^-$, NO$_3^-$, and NO$_x^-$ were quantified using chemiluminescence as described (Ignarro 1990, 1993; Ma et al., 1999). The skin together with subcutaneous tissue was examined in meridian regions from PC 2 to PC 6, BL 36 to BL 57, and CV 3 to CV 22 and their control tissues. Tissue samples from the skin regions, gracilis muscle, superior mesenteric artery, aorta, coronary artery, and pulmonary vessels were immediately cut into slices (0.5 × 0.5 mm) with a tissue chopper. Micropunches of the hypothalamus (AH), the posterior hypothalamus (PH), the nucleus tractus solitarii (NT), and the lateral reticular nucleus (LN) were identified and isolated bilaterally from two adjacent slices under a microscope.

Measurements of NO$_2^-$, NO$_3^-$, and NO$_x^-$ concentrations were conducted in a blinded manner. Briefly, each sample was weighed and was homogenized manually in a 1:10 w/v ratio of methanol. The mixture was centrifuged at 40,000 × g for 10 min in an Eppendorf 5415C microcentrifuge at 4°C. A 100-μL aliquot of the supernatant was injected onto a chemiluminescence NO analyzer (Dasibi, Glendale, CA) and the concentrations of NO$_2^-$ and NO$_3^-$ were determined by chemiluminescence as described (Ignarro et al., 1993; Ma et al., 1999). Samples containing NO$_2^-$ and NO$_3^-$ were reduced to NO gas, which can be quantified by the chemiluminescence detection device after reaction with ozone. Refluxing 1% potassium iodide in glacial acetic acid causes a rapid one-electron reduction of NO$_2^-$ to NO gas and this acidification/reduction solution was used to determine NO$_2^-$ concentrations. NO$_x^-$ concentrations were measured by refluxing 1.5 mM vanadium (III) chloride in 2 M HCl because refluxing acidic vanadium (III) can quantitatively reduce both NO$_2^-$ and NO$_3^-$ to NO gas. Values for NO$_2^-$ and NO$_x^-$ were quantified and values for NO$_3^-$ were calculated by subtracting NO$_2^-$ from NO$_x^-$ values. The NO analyser was calibrated with known concentrations of NO$_2^-$ (NaNO$_2$). The linearity of the standard curves was made each day, which encompassed the range of nitrogen oxides produced by the experimental samples. The quantitative analyses was based on measurements of peak areas of the standard samples. The lower limit of the detection of this assay was 0.1 pmol of NO. Protein was assayed in 200 μL of homogenate from the remaining tissue same
as used in the measurements of NO, NO$_2^-$, NO$_3^-$, and NO$_x^-$ concentrations in skin region were expressed as pmol/mg protein and were compared with groups.

**Immunoblot analysis**

The various samples of the skin and subcutaneous tissue containing meridian lines with LSRP and control regions without LSRP were isolated using methods as described above. Proteins were quantified using the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to manufacturer’s specifications and using bovine serum albumin as a standard.

Protein samples of the same concentration were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The resultant gels were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) at a constant voltage of 80 V for 1.5 h, in a wet blotting system (Bio-Rad, Richmond, CA). Nitrocellulose membrane containing the transblotted proteins were pretreated in blocking buffer [5% (w/v) nonfat milk proteins in 50 mM Tris buffer, pH 7.4] at room temperature for 2 h to block nonspecific binding. The blots were then incubated for 1 h with 1:1000 dilution of either a rabbit polyclonal nNOS or eNOS antibody (Transduction Laboratories, Lexington, KY) in Tris buffer containing 0.5% milk, pH 7.4 (Gozal et al., 1996; Ma et al., 2000). After this treatment, the membranes were washed extensively with Tris buffer and were then incubated with the appropriate peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) diluted in 1% (w/v) Marvel in phosphate-buffered saline (PBS) for 1 h at room temperature. The blots were washed again for 1 h and developed with enhanced chemiluminescence (ECL) kits (Amersham, Arlington Heights, IL), as described in the manufacturer’s instructions.

Western blot analysis for protein concentration in each region was quantified using an Eagle Eye™ II Densitometry (Stratagene, Cambridge, UK) to measure the density per area (Gozal et al., 1996; Ma et al., 2000). Band intensities were examined by submitting each luminol-reacted membrane to several x-ray exposure times and selecting the one(s) falling within the film response ranges. The films were then scanned and each band density was evaluated. The quantitation was performed in a blinded fashion for sections of skin of all subjects and compared in different groups.

**Statistical analysis**

Results were expressed as mean ± standard error mean (SEM). Six to eight rats were used for each defined group. Analysis of variance (ANOVA) and Fisher’s LSD was used to analyze significant difference, with $p$-values <0.05 considered significant.

**RESULTS**

**Measurements of skin electrical resistance of acupoints**

The LSRP were performed in fourteen Sprague–Dawley rats anesthetized with pentobarbital sodium (50 mg/kg, i.p.). LSRP on the skin surface along PC 2 to PC 6, BL 36 to BL 57, and CV 3 to CV 22, and GV 2 to GV14 were tested in each rat and the meridian lines were marked. The current (μA) on LSRP in response to a pulse electrical stimulation in rats was 8.5 ± 1.9 (Mean ± SE) in BL and GV, and 10.6 ± 2.2 in PC and CV compared with 3.5 ± 1.7 on control regions ($p < 0.05$). LSRP were detected by the stimuli applied to the points which causes more than 50% increase in current value.

LSRP can be detected reproductively in different animals. Most points were found to be distributed symmetrically and bilaterally. The measurements may not cover all LSRP on the rat’s skin as some points may have not been thoroughly detected. Some LSRP on the experiments exhibited increasing current of <50% in response to the stimuli, which were not included due to the abundance and closeness of the sites. These findings are consistent with the results of previous studies of the rat LSRP and meridians (Zhu and Hao, 1989; Chiou et al., 1998).

**Quantification of NO metabolites in skin meridian regions**

NO metabolites, NO$_x^-$, NO$_2^-$, and NO$_3^-$ were measured in skin tissues, brain regions
and blood vessels \((n = 6-8)\). The skin together with subcutaneous tissue was isolated in meridian regions from PC 2 to PC 6, BL 36 to BL 57, and CV 3 to CV 22 for assay of NO metabolites. Figure 1 shows the \(\text{NO}_2^+\), \(\text{NO}_3^-\), and \(\text{NO}_x^-\) concentrations in the three skin meridian regions compared to control skin tissues, which were obtained close to related meridian line without containing LSRP. \(\text{NO}_x^-\) and \(\text{NO}_3^-\) levels were consistently increased in three meridian skin tissues (Fig. 1, middle and bottom panels). \(\text{NO}_x^-\) and \(\text{NO}_3^-\) concentrations were higher in the CV meridian skin tissues \([45 \pm 8\% \text{ (mean } \pm \text{ SE}) \text{ and } 43 \pm 7\%]\), in the BL \([47 \pm 7\% \text{ and } 51 \pm 9\%]\), and in the PC \([47 \pm 8\% \text{ and } 45 \pm 6\%]\) than those in their control skin tissues \((n = 6-8, p < 0.05)\). \(\text{NO}_2^-\) concentrations were not significantly different between three meridian regions and controls (Fig. 1, top panel). NO metabolites were consistently higher in three meridian skin regions, which suggest that NO contents are increased in acupoints/meridians.

We also tested the \(\text{NO}_x^-\) levels in the skin tissues compared to the brain regions and blood vessels (Fig. 2). \(\text{NO}_x^-\) concentrations were not significantly different among brain regions (the AH, PH, NT, and LN) and blood vessels (gracilis muscle, superior mesenteric artery, aorta, coronary artery, and pulmonary vessels). \(\text{NO}_x^-\) concentrations were consistently higher in skin tissues of three meridian regions compared with control skin tissues. \(\text{NO}_x^-\) concentrations in skin tissues are more than 2- to 3-fold greater than the values in the brain regions and blood vessels (Fig. 2, \(p < 0.05\)). The data show that high concentrations of NO are predominantly located in skin tissues compared with brains and peripheral vessels.

**Immunoblot analysis**

To learn whether nNOS system is associated with NO concentrations in the areas, nNOS and eNOS immunoreactive proteins were examined in meridian skin regions and control skin tissues. The skin and subcutaneous tissues containing acupoints/meridians with LSRP on skin surface in BL, PC, and GV meridians and control regions without LSRP were isolated in six rats. Western blots of skin tissue homogenates with nNOS antibody displayed a relative abundance of nNOS protein expression in GV and BL meridian regions compared with their control areas (Fig. 3, top panels). Figure 3, bottom panels shows that there are significant increases of nNOS protein levels in the GV, BL, and PC meridian regions as compared with...
the control tissues \((p < 0.05)\). Although little or small amount of eNOS protein was observed in skin tissue homogenates, it was not different among the three groups of meridian skin regions compared with the controls (data not shown). The results demonstrate that nNOS protein levels are consistently increased in the skin tissues containing acupoints/meridians.

**DISCUSSION**

We examined the concentrations of NO\(_3^-\), NO\(_2^-\), and NO\(_x^-\) in the meridian skin regions containing LSRP, detected by electrical methods compared with control areas in rats. The protein levels of nNOS and eNOS were also quantified using Western blots in the skin meridian regions compared with the control skin tissues. The major new findings of this study are: (1) NO\(_3^-\) and NO\(_x^-\) concentrations are higher in the three meridian skin regions containing LSRP; (2) NO content is much greater in skin tissues than in brain regions and blood vessels; and (3) The protein levels of nNOS are consistently enhanced in the three meridian skin regions. This is the first evidence showing that NO metabolites are consistently increased in three meridian skin regions, which suggest that NO contents are increased in acupoints/meridians. The data show that high concentrations of NO are predominantly located in skin tissues compared to brains and peripheral vessels. The results also demonstrate that nNOS protein levels are increased in the acupoints/meridians, which supports our NO assay data and suggests that neuronal NOergic systems may serve as one resource, at least in part, for enhanced NO contents in the acupoints/meridians.

Early investigators (1976–1979) demonstrated that acupoints possess the characteristics of low impedance, high electric potential, and hypersensitivity to noxious stimulation (Ahu, 1981; Fraden, 1979; Luciani, 1978; Reichmanis et al., 1976). Topographies of LSRP have been established in both humans and rats (Ahu, 1981; Zhu and Hao, 1989; Chio et al., 1998). Luciani (1978) has indicated that low impedance acupoints on the skin may reflect the variation in anatomical concentration of nerve fibers beneath the skin and represent areas of potentially high neuronal activity. Investigators noted that, at the light microscopic level, the numbers of nerve bundles, nerve fibers, and nerve endings were higher in the skin under the low impedance line than those in their adjacent control areas in both patients and rats (Chan et al., 1998; Zhu and Hao, 1989). The results of the present study also show that LSRP are detected by their low electrical resistance characteristics in the BL, PC, CV, and GV meridians in rats. These locations closely correspond to the acupoints of animals described in the Chinese classical topography, which were evolved from comparative anatomy based on technical development in classical human topography (Ahu, 1981; Zhu and Hao, 1989). These results support the previous findings that acupoints possess the characteristics of low electrical resistance and demonstrate an accompanying enhanced nNOS-NO in the acupoints/meridians. The data also show that NO
contents are much greater in skin tissues than in brains and blood vessels, which suggests a potentially important role for NO generation in the represented areas of the skin, and NO may be chemically important in the acupoints/meridians. Recent studies have demonstrated that the epidermis and the outer root sheath exist both nNOS immunoreactivity and NADPH diaphorase reactivity (Dippel et al., 1994). Dermal microdialysis has revealed that NO levels and other chemical messengers in human skin increase during the inflammatory weal and flare response (Clough et al., 1998a, 1998b). The present data suggest that increased NO content in the meridians/acupoints is associated with an enhanced nNOS but not eNOS. The results suggest that elevated NO in the acupoints/meridians is independent to endothelial NO synthesis and is involved in neuronal NOergic systems, which support that acupoints/meridians may lie close to peripheral neural components. However, our data also demonstrate that NO content is much greater in skin regions than in brain tissues. Because brains contain the highest nNOS activity in the body, it is likely that the higher NO content in skin tissues may be generated from other resources in addition to being synthesized by an enzyme known as nNOS. Recent studies have reported that NO is generated on the surface of the skin by chemical reduction of nitrate to nitrite and skin nitrate is present in sweat (Weller et al., 1996). Nonenzymatic NO production has been demonstrated on the surface of the skin, in the stomach, in the ischemic heart, and in infected nitrite-containing urine (Duncan et al., 1995; Weitzberg and Lundberg, 1998; Weller et al., 1996). Sweat gland innervation for several neurotransmitters and neuropeptides have been shown in rat skin, and rat sweat secretory response is elicited by stimulation of the sciatic nerve (Bharali et al., 1988; Landis et al., 1988; Stevens and Landis, 1987). The result of the present studies show that NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{\textit{x}}\textsubscript{−}\textsuperscript{−} but not NO\textsubscript{2}\textsuperscript{−} concentrations are high in the acupoint/meridian skin. Our findings would be consistent with this possibility that NO is generated in the skin surface by reduction of sweat nitrate, and suggest that nonenzymatic NO production may be involved in higher NO content in the skin acupoints/meridians. However, the precise resources and mechanisms are still unclear. A more sophisticated approach including assay of NO content in living tissues compared with dead animals would be required to address this issue. Despite these limitations, our NO content assays and immunoblot analysis results consistently suggest an enhanced nNOS-NO level in the acupoints/meridians.

With regard to the potential role of NO in biophysical characteristics of skin acupoints/meridians, it has been demonstrated that NO serves as a messenger in the neurons,
much like a neurotransmitter with a widespread signaling mechanism and function (Bredt and Snyder, 1992; Ma et al., 1995; Moncada and Higgs, 1991). Our previous results show that nitroglycerin, a NO donor, increases release and synthesis of norepinephrine (NE) in the central and peripheral nervous system, suggesting that NO produces noradrenergic activation in neurons (Ma and Long 1991a, 1991b). NO stimulates the release of NE from neurons in microdialyzed rats and in vitro (Lonart et al., 1992; Satoh et al., 1996). Several groups of the studies have reported that stimulation of sympathetic pathways either locally or systemically lowers the skin resistance levels (Korr et al., 1958). In contrast, interruption or retardation of the flow of impulses over sympathetic pathways to a given area of skin causes marked elevation of resistance in that area either by pharmacological blockade, local anaesthetic, peripheral nerve lesions, or severance of pre- or postganglionic pathways (Korr et al., 1958; Egyed et al., 1980; Smith et al., 1988). It is well established that the actual value of skin electrical resistance depends upon the activity of the sympathetic nervous system and is a direct result of changes in the degree of skin sweating (Korr et al., 1958; Smith et al., 1988). The results of these studies demonstrate that nNOS-NO levels are higher in the acupoints/meridians accompanied with low skin resistance. Enhanced NO may evoke the release of NE in sympathetic nerve terminals, which plays an important role in response to skin electroconduction and reduces skin resistance. NO may also serve as a messenger for sympathetic nerve activation in the dermal neurons, which might be associated with their biophysical characteristics.

In summary, NO contents and nNOS protein levels are consistently increased in three skin acupoints/meridians associated with low electric resistance in rats. NO\textsubscript{3} and NO\textsubscript{2} concentrations are 2- to 3-fold greater in skin tissues than those in brains and blood vessels. Thus, enhanced NO content in the skin acupoints/meridians may be generated from nonenzymatic NO production in addition to neuronal NO synthesis catalyzed by nNOS. These data suggest a potentially important role for NO as a messenger for sympathetic nerve activation in the dermal neurons, which may mediate meridian/acupoint functions including low electric resistance.

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