Modulation of microvascular function following low-dose exposure to the organophosphorous compound malathion in human skin in vivo

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Boutsiouki, Paraskevi, and Geraldine F. Clough. Modulation of microvascular function following low-dose exposure to the organophosphorous compound malathion in human skin in vivo. J Appl Physiol 97: 1091–1097, 2004; 10.1152/japplphysiol.00123.2004.—This study investigates whether malathion, a widely used organophosphate insecticide, has its effects on cutaneous vasculature in healthy human volunteers through its anticholinergic activity or through the modulation of other, noncholinergic pathways. Acute, low-dose exposure to malathion (10 mg/ml for 5 h under occlusive dressing) caused a significant increase in cutaneous blood flux, monitored by using laser-Doppler flowmetry and imaging. It had little effect on tissue levels of ACh, nitric oxide, and histamine assayed in dermal dialysate collected from malathion-exposed and control-treated skin. The duration of the cutaneous vascular response to exogenous ACh (2%) delivered by iontophoresis was significantly enhanced by pre-exposure to malathion, both <1 h after its removal and 24 h later (P < 0.001). At <1 h, the time to 50% decay of the response was 24 ± 4 and 50 ± 8 min in control and malathion-treated skin, respectively. Malathion also enhanced the size and duration of the axon reflex-mediated vasoresponse to ACh. The increase in blood flow to malathion and the endothelium-mediated response to exogenous ACh, both in the presence and absence of malathion, were attenuated by pretreatment of the skin with atropine and local anesthesia (P < 0.01). We conclude that short-term exposure to a single low dose of malathion causes prolonged modulation of the physiological function of the cutaneous vasculature and that this is, in part, through its action on acetylcholinesterase at both neuronal and nonneuronal sites.

endothelial cells; acetylcholine; vasodilatation

ORGANOPHOSPHOROUS COMPOUNDS (OP) are well recognized to have the potential to cause severe, acute toxicity through the phosphorylation of serine residues of acetylcholinesterase (AChE) and the subsequent accumulation of ACh. There is increasing awareness that repeated low-level exposure to OP can also have adverse effects on health, including the modulation of inflammatory and T-cell-mediated immune responses (21). Many of these effects are on nonneuronal cells. They can occur at or below the concentrations of OP required to inhibit AChE (i.e., below the cholinergic threshold) and may thus be through mechanisms other than the direct inhibition of AChE.

In nonneuronal cells, there is evidence for a wide range of novel target proteins that are more sensitive than AChE to moderate- or low-dose exposure to OP (21). It has been shown that such exposure can result in phosphorylation of proteases, esterases, and other cell signaling molecules, as well as have effects on cytoskeletal elements, including the nitric oxide synthase (NOS) Ca-calmodulin complex (20). Furthermore, both IgE-dependent and -independent activation of human leukocytes by low-dose OP has been demonstrated (22, 23). Low-level OP exposure has also been shown to cause cytotoxicity (3) and perturbations in cell membrane structure (30) and muscarinic receptor activity (11) in some cell prepreparations. The potential for long-term modulation of nonneuronal cell function and consequent effects on human health is thus significant and wide ranging.

The skin is one of the major routes for the absorption of OP, and a number of nontoxic dermatoses have been reported following chronic exposure and environmental contamination. The most common are allergic and irritant dermatitis (4) and urticaria (27). Both are characterized by transient increases in vascular perfusion (vasodilatation) and permeability (edema) of the blood vessels of the upper dermis. The mechanisms underlying OP-induced cutaneous reactions are unclear, and their relevance to human disease is yet to be elucidated.

Our laboratory has previously shown, using the in vivo sampling technique of microdialysis, that, when the OP malathion [S-(1,2-dicarbethoxyethyl) O,O-dimethyl-phosphorothioate] is applied to the skin of healthy human volunteers, it is rapidly absorbed into the dermis and that, after even brief (5 h) exposure, the concentration measured in the skin (60–600 ng/ml) is sufficient to cause a cutaneous reaction that persists for up to 24–48 h (6). One of the major characteristics of this exposure is a sustained increase in skin blood flux in the region of application.

The aim of the present study was to investigate the mechanisms by which low-level exposure to malathion has its effect on the cutaneous vasculature in human skin in vivo and, specifically, to test the hypothesis that the cutaneous vascular response to malathion is due to its anticholinergic activity. To explore this, we have investigated modulation by malathion of the cutaneous vascular response to ACh. We have also investigated whether malathion can modulate other, noncholinergic pathways in the skin to increase vascular hypersensitivity.

MATERIALS AND METHODS

Study Population

The study was performed on 43 healthy volunteers (28 women and 15 men), aged between 18 and 45 yr (mean age 28 ± 3 yr). The study was performed with local medical research ethics committee approval (LREC 301/98 and 138/01) in the Clinical Research Facility of Southampton General Hospital. All volunteers gave informed, signed consent. Volunteers with dermatological problems, allergic disease, or...
car</p> cardiovascular disorders or those on prescribed medication were excluded from the study. All subjects were normotensive and non-smokers, and all were asked to refrain from vasoactive medication, alcohol, and caffeine 12 h before the study. Volunteers were acclimatized to 22–23°C for 30 min before the start of the experiment, which was performed with the subjects lying in the supine position with their arms at heart level.

**Application of Malathion**

Malathion (malathion pestanal, Riedel-de-Haen, RDH Laborchemikalien, Sigma) dissolved in ethanol (40 μl malathion to 200 μl ethanol) was mixed with 5 g of an aqueous-based gel (Aquagel, Adams Healthcare, Leeds, UK) to give a malathion preparation of 10 mg/ml. Aquagel (5 g), mixed with 200 μl of ethanol, was used as the vehicle control. Both gels were prepared on the day of use. Malathion or its vehicle control (0.5 ml) were applied in a randomized manner to four 2 × 2.5-cm areas of the ventral surface of the nondominant forearm, each separated by at least 5 cm. The gel was contained within a drug well under occlusive dressing (3M Tegaderm, Astra Pharmaceuticals, Kings Langley, Herts, UK) and left in contact with the skin for a period of 5 h. Experiments were performed immediately (<1 h) and 24 h after removal of the malathion-containing gel. Experimenter and volunteers were blinded to the content of the gel. The gel was removed after 90 min, and local analgesia was tested by light touch and pin prick.

**Delivery of vasoactive agonists and antagonists.** Drugs were delivered across the skin to the malathion and control-treated sites by iontophoresis by using a battery-powered, constant-current iontophoresis chamber (3M Tegaderm, Astra Pharmaceuticals, Kings Langley, Herts, UK) and their vehicle control (distilled water) were delivered to both sites of the iontophoresis chamber (MIC 1, Moor, Axminster, UK). The Perspex iontophoresis chamber (diameter 10 mm, area 0.7 cm²) with a platinum internal wire electrode was fitted with a laser-Doppler flow probe (DPIT, Moor) to monitor blood flow and skin temperature continuously within the area of iontophoresis (DRT4, Moor). Vasoactive agonists ACh (Sigma; 2%; anodal current) and sodium nitroprusside (SNP; David Bull Laboratories DBL, Warwick, UK; 1%; cathodal), and their vehicle control (distilled water) were delivered to both malathion and control pretreated skin by using successive charges of 1 mC (100 μA, 10 s), 2 mC (200 μA, 10 s), 4 mC (200 μA, 20 s), and 8 mC (200 μA, 40 s), preceded by a 20-s baseline period.

The role of muscarinic receptors in the mediation of the malathion-induced vascular response was investigated by using atropine (10 mM atropine sulfate, Sigma) or its vehicle control (distilled H₂O) delivered to areas of skin previously exposed to 5-h malathion or its vehicle control under occlusive dressing (3M Tegaderm, Astra Pharmaceuticals, Kings Langley, Herts, UK) at a rate of 5 μl/min. The dialysate was collected over timed periods before and during exposure to malathion or its vehicle control into plastic vials and stored at −20°C before assay. NO, present as NO₂ in the dialysate solution, was assayed by using a gas-phase chemiluminescence method (Sievers NOA 280, Sievers, Bolder, CO), sensitive to 2 pmol. The in vitro dialysis efficiency of the probes for NO has previously been shown to be >80% when perfused at a rate of 5 μl/min. Histamine was assayed in 10-μl dialysate samples by using a commercial immunoassay (Immunotech) sensitive to 0.5 nM and with a coefficient of variance for the assay of 6.2%.

**Data Acquisition and Statistical Analysis**

All cutaneous blood flow data were acquired by using the manufacturer’s data-acquisition software (Moor). Mean blood flux within a defined region of interest was calculated by using the manufacturer’s software (Moor, SLDI version 3.01) and expressed in laser-Doppler PU. The regions of interest used were 1) the 2 × 2.5-cm area of malathion/vehicle application, 2) a 1-cm-diameter circle directly over the site of the iontophoresis chamber, and 3) the area over which a wider spread flare response developed outside the area of iontophoretic drug delivery (see Fig. 1). This latter area was usually contained within that of the 2 × 2.5-cm area of exposure to malathion. To calculate the relative increase in mean blood flux in response to iontophoretic delivery at malathion/vehicle-treated sites, mean flux within area 1 was subtracted from those estimated in areas 2 or 3. All SLDI images were calibrated by using a 2-cm scale, and this was used to calculate the area of each response.

The comparisons of basal vs. postmalathion exposure blood flux were done by using a Student’s t-test for paired data. The dose-response curves were analyzed by using an ANOVA for repeated measures. For all iontophoresis protocols, a separate analysis was done of each recording site (direct and axon-reflex mediated). Dialysate concentrations of mediators were compared by using Student’s t-test for paired data. All volunteers acted as their own controls. All data are presented as means ± SE. Statistical significance was acknowledged if the probability of a type 1 error was <5% i.e., P < 0.05.

**RESULTS**

**Effects of Malathion on Skin Blood Flow**

Malathion applied to the skin of the forearm for 5 h under occlusive dressing increased blood flux within the region of application from a baseline of 108 ± 6 to 290 ± 40 PU (P < 0.001; 22 sites in 11 volunteers). There was a small but not
significant increase in blood flux at vehicle control-treated sites from a baseline value of 107 ± 5 PU to one of 130 ± 8 PU measured at 22 sites in 11 volunteers. Pretreatment with atropine but not its vehicle (distilled H2O) significantly attenuated the malathion-induced increase in blood flux within the area of iontophoresis (48 ± 5%, n = 5, P < 0.05). It had little effect on blood flux outside the area of iontophoresis. The same dose of atropine, when tested for its effectiveness 30 min and 5 h after iontophoretic delivery to unexposed skin, significantly attenuated the direct response to ACh by 45 ± 9 and 20 ± 5%, respectively (n = 5, P < 0.05).

Modulation by Malathion of the Vascular Response to Exogenous ACh

To explore whether the effect of malathion on skin blood flow was a direct consequence of its inhibition of AChE and the subsequent increase in tissue ACh, we investigated whether the cutaneous vascular response to exogenous ACh was modulated in malathion-exposed skin.

Less than 1 h after malathion exposure. Iontophoretic delivery of ACh within 1 h of the removal of malathion or its vehicle caused a significant increase in blood flux at both the malathion and control-treated sites (Fig. 1). Correction of the data to a single baseline (control site) shows the vertical displacement of the responses at the malathion-treated sites to be a result of the malathion-induced increase in basal blood flux (Fig. 2A) and that the dose-blood flux relationship was not significantly different at the two treatment sites. However, repeat measurement of blood flux for 30 min after iontophoretic delivery of ACh showed the ACh-induced response to last longer and resolve more slowly at malathion-treated sites compared with control (Fig. 3A). The time to decay to 50% of the peak response was 25 ± 4 and 50 ± 8 min in control and malathion-treated skin, respectively (n = 11, P < 0.001). Malathion had little effect on the concentration-dependent blood flux response to SNP or on the response to vehicle control (Fig. 2, B and C). The time to decay to 50% of the peak response to SNP in the presence of malathion (32 ± 10 min) was not significantly different from that at control-treated sites (28 ± 7 min).

To investigate whether malathion had an effect on sensory nerves in the skin, we also investigated the neurogenic flare response to ACh. As in previous studies (2, 5), iontophoresis of ACh resulted in an area of increased blood flux that extended outside the 0.7-cm² area of the iontophoresis chamber (Fig. 1). The area of the neurogenic response (excluding the area of the chamber) at the end of the ACh iontophoresis protocol was 1.6 ± 0.4 cm² at control-treated sites and 2.9 ± 0.7 cm² at malathion-treated sites (P < 0.001, n = 11). The increase in mean blood flux above baseline within this area was also significantly greater in malathion-exposed skin (434 ± 21 vs. 360 ± 29 PU, n = 11, P < 0.001). The time to decay to 50% of peak flux response was 15 ± 4 and 49 ± 6 min in control and malathion-treated skin, respectively (n = 11, P < 0.01). The resolution of the area of the flare response from peak was also slower at malathion-treated sites (n = 11, P < 0.05; Fig. 4).

Fig. 1. Scanning laser-Doppler images of blood flux before and after iontophoretic delivery of ACh (2%) to the skin, at sites previously exposed for 5 h to malathion (10 mg/ml) or aquagel control under occlusive dressing. White square shows the area of treatment; white circle shows the area of iontophoresis. Measurements of mean blood flux were made within the circle (direct effect of ACh) and outside it (neurogenic flare response). PU, perfusion units.
Pretreatment with topical local anesthetic significantly reduced the area of the ACh-induced flare by 50% (from 2.9 ± 0.7 to 1.2 ± 0.3 cm²) at the malathion-treated and 30% (from 1.6 ± 0.4 to 1.2 ± 0.2 cm²) at vehicle control-treated site (n = 6, P < 0.05).

Twenty-four hours after malathion exposure, the ACh-induced increase in blood flux within the area of iontophoresis 24 h after malathion exposure was similar to that measured in the same individuals at 1 h at both malathion (598 ± 46 PU) and vehicle control (580 ± 105 PU) treatment sites (n = 5).

Fig. 2. Cutaneous vascular response to ACh (2%; A), ACh vehicle (distilled H₂O; B), and sodium nitroprusside (SNP 1%; C) delivered by iontophoresis by using a current protocol of 1 mC (100 μA, 10 s), 2 mC (200 μA, 10 s), 4 mC (200 μA, 20 s), and 8 mC (200 μA, 40 s), preceded by a 20-s baseline measurement. Skin was pretreated for 5 h with either malathion (10 mg/ml; ●) or aquagel control (○). Blood flux was measured by using a laser-Doppler flow probe mounted in the iontophoresis chamber and normalized to the baseline flux measured at the aquagel control-treated site. The vertical displacement of the response curves is due to the malathion-induced increase in blood flux before iontophoresis. Values are means ± SE from 11 volunteers.

Fig. 3. Resolution of the ACh-induced increase in skin blood flux at malathion (10 mg/ml, 5 h; ●) and aquagel control-treated sites (○) <1 h (A) and 24 h (B) after exposure of the skin. Mean blood flux within the area of iontophoresis (laser-Doppler PU) was measured at intervals up to 30 min after the end of the iontophoresis protocol (see Fig. 1 and text for details). Values are means ± SE. * P < 0.001 (single time point malathion vs. vehicle; Student’s t-test for paired data; n = 11). The relative slopes of the recovery slopes at malathion and control-treated sites are −0.11 ± 0.04 and −0.24 ± 0.03 min⁻¹ at <1 h (ANOVA, P < 0.001, n = 11) and −0.14 ± 0.04 and −0.27 ± 0.06 min⁻¹ at 24 h (ANOVA, P < 0.01, n = 5).
Detection of Vasoactive Mediators in Dermal Dialysate

However, as seen at <1 h, the resolution of the direct response was slower at the malathion-treated sites (Fig. 3B) and did not differ significantly from those measured <1 h after removal of the malathion.

At 24 h after exposure to malathion, neither the increase in mean blood flux in the ACh-induced flare response outside the area of iontophoresis nor the area were significantly different at malathion (385 ± 33 PU and 2.0 ± 0.7 cm²) and vehicle control-treated sites (305 ± 77 PU and 1.3 ± 0.4 cm²; n = 5). However, the duration of the flare response at 24 h remained prolonged at the malathion-treated sites and similar to those measured at <1 h (Fig. 4).

Detection of Vasoactive Mediators in Dermal Dialysate

The concentration of NO, measured as nitrite in dialysate collected immediately after the 5-h exposure period, was not significantly different in the erythematous malathion-treated skin (1.5 ± 0.2 μM) and vehicle control-treated skin (1.25 ± 0.2 μM; n = 9). Dialysate histamine concentration was not significantly higher in malathion-exposed skin compared with vehicle control, and both remained close to basal levels measured at the same site before exposure (4–60 nM). These levels did not differ significantly from those reported previously in resting skin (19).

DISCUSSION

Malathion is a commonly used pesticide and, because of its relatively low toxicity to mammals, is licensed for domestic use as an insecticide and clinically as a human acaricide for application to the skin. From our laboratory’s previous studies, we know that, even during brief exposure, malathion penetrates the skin in sufficient amounts to cause a long-lived erythematous response (6). In the present study, we provide indirect evidence that this response is a result of malathion acting as a long-lasting anticholinesterase. We have shown that 5-h exposure to a 10 mg/ml solution of malathion significantly enhances the duration of cutaneous vascular response to exogenous ACh and, furthermore, that the effects of acute low-dose exposure to malathion on cutaneous vasoreactivity are still evident 24 h after its removal from the skin.

ACh plays an important role in the regulation of vascular tone via its effects on both cutaneous blood vessels (5, 17, 18) and nerves (2). It also has the potential to play an important part in host defense mechanisms as well as in tissue repair and remodeling (29). There is increasing evidence that cholinergic systems are not confined to the nervous system and that a number of nonneuronal cells in the skin, including endothelial cells, leukocytes, mast cells, and keratinocytes, express the necessary essential components (16). This raises the possibility that ACh may play an autocrine or paracrine role in mediating cell signaling in nonneuronal cells and that inhibition of AChE after exposure to OP, and the subsequent accumulation of ACh, may result in a modulation of these processes.

The primary aim of our study was to investigate whether malathion had its effect on the skin vasculature via the inhibition of AChE to cause an increase in local tissue levels of ACh. As microdialysis recovery of ACh from skin proved problematic, we employed an indirect method whereby exogenous ACh was used to test the activity of endogenous AChE in malathion-exposed and control-treated skin. Under normal conditions, intradermal injection, iontophoresis, or microdialysis delivery of ACh causes a dose-dependent, short-lived dilatation, which resolves within 10–20 min (2, 5, 17, 18). In the presence of malathion, the duration of this local response was significantly prolonged and, in some individuals, took up to 1 h to resolve. Whether this represents a considerable prolongation of the vasodilatory response to ACh through continued receptor occupation or is a consequence of the time needed to recover from a heightened initial response cannot be determined from this study. The extended duration of the ACh-induced vasodilatation is, however, similar to that reported in patients with chronic fatigue syndrome, in whom there is evidence of peripheral cholinergic dysfunction (15). In these patients, Khan et al. (15) reported the response to ACh to be significant and the time to decay to 50% peak to be ~40% longer than in healthy controls. They were, however, unable to demonstrate a similar phenomenon in patients with Gulf War Syndrome or in agricultural workers exposed to OP-containing pesticides (14).

It has previously been shown that edrophonium given via the brachial artery causes no change in resting forearm blood flow. However, given in conjunction with ACh, it does cause a 10-fold reduction in the dose of ACh that was required to cause...
a maintained increase in forearm blood flow (7). Oral pyridostigmine bromide has also been shown to reduce skin blood flow in humans and to increase the threshold for initiation of cutaneous vasodilation (28). Together, these data provide a link between vascular cholinesterase activity and the dynamics of ACh-induced blood flow responses. Our data suggest that malathion, under the experimental conditions used, has its effect to prolong the cutaneous vascular response through inhibition of AChE to slow the enzymatic degradation of ACh and thus to extend binding of ACh to muscarinic receptors on the endothelium. Moreover, preliminary experiments using human dermal microvascular endothelial cells in vitro have confirmed that the active metabolite of malathion, malafoxon, can dose dependently inhibit endothelial AChE activity over a dose range of 10^{-7} \text{-} 10^{-3} \text{ M} (10, 24).

The similarity in the magnitude of blockade by atropine of the erythematous response to malathion and exogenous ACh is further evidence that the malathion-induced erythema is mediated by ACh via atropine-sensitive muscarinic receptors (12). However, this pharmacological approach cannot rule out the possibility that malathion may also interact directly with cholinergic muscarinic receptors (11, 31). The location of these receptors in the skin has yet to be determined. We found no evidence that endothelial cell and/or smooth muscle cell reactivity was modified by our experimental conditions. The vasodilatory response to SNP and to a range of other vasoactive mediators (data not shown) was similar in both acute 5-h malathion-exposed and vehicle control-treated skin.

As well as inducing an endothelium-dependent increase in cutaneous blood flow, ACh can also activate nociceptive C fibers to cause an axon-reflex-mediated vasodilatation in the skin (2, 5). Preexposure of the skin to malathion significantly increased the size and duration of this flare response, which is indicative of an altered nociceptive function (32). Whether the malathion-induced augmentation of the response is a direct result of the accumulation of excess ACh or is secondary to the release of other mediators of the neurogenic flare has yet to be fully resolved. Previous studies have shown that malathion causes a dose-dependent mast cell degranulation and histamine release, maximally detectable in serum between 4 and 8 h after acute administration (23). We, however, found no increase in tissue levels of histamine in microdialysate from skin exposed to malathion for up to 5 h. Thus our data provide further evidence that the response that we observe to low-dose exposure to malathion was not through the modulation of a classical inflammatory cascade.

We were unable to detect an increase in tissue levels of NO in malathion-exposed skin. This was somewhat unexpected as one of the major mediators of the ACh-induced vasodilatation in skin is NO (5, 17). It is possible that the ACh-induced NO production declines during prolonged dermal exposure to excess ACh and that the OP-induced erythema is maintained by other endothelium-derived factors (13, 17). Alternatively, our apparent failure to measure an increase in tissue levels of NO by dialysis may be due to an increase in the extraction of these highly labile solutes from the tissue space consequent to the malathion-induced increase in local blood flow (9). Interestingly, OP have been shown in vitro to suppress NO production (1) and to inhibit NOS activity, possibly through an interaction with Ca^{2+}-calmodulin on which NOS is dependent (20). Thus it cannot be ruled out that our failure to recover NO from malathion-exposed skin is the result of a local inhibition of NOS by OP.

Using our protocol, we were able to investigate the longer term effects of a single low dose of malathion on the skin microvasculature. The results indicate that, even 24 h after its removal from the skin, malathion can effectively modulate the response to exogenous ACh. It can be argued that the inhibitory action of malathion on AChE or its action on cholinergic receptors persists for 24 h. This is supported by the demonstrable erythema that persists for \( >24 \text{ h} \) after experimental 5-h exposure to malathion and the long-lived effects that follow therapeutic and occupational exposure (25, 26). These findings are perhaps not unexpected, as malathion is thought to be an irreversible inhibitor of AChE and reactivation of the enzyme is only possible by de novo synthesis and/or oxime treatment. This raises important questions as to whether the effects of malathion, via its interaction with AChE and other serine esterases, may also be associated with an increased susceptibility to inflammatory challenges.

In conclusion, this study provides evidence for the prolonged modulation of the physiological function of the cutaneous vasculature following short-term exposure to a single low dose of malathion in vivo. Furthermore, it suggests that the skin and its vasculature can be used as a model tissue in which to study the potential for effects of both acute and chronic exposure to OP at low levels, which do not cause the cholinergic symptoms associated with acute high-level exposure.

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