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Ivermectin: concentration-dependent effects on adenosine triphosphatases in adult worms of Onchocerca volvulus

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Ivermectin: concentration-dependent effects on adenosine triphosphatases in adult worms of *Onchocerca volvulus*

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Abstract

The effect of increasing concentrations of ivermectin on adenosine triphosphatase (ATPase) activity was investigated in adult worms of *Onchocerca volvulus*. Mean Mg- and Na,K-ATPase activities decreased significantly \((F\) ratio = 29.82, \(P < 0.01\) and \(F\) ratio = 28.54, \(P < 0.01\), respectively) with increasing concentrations of ivermectin (0–100 ng/ml) in the female worms. When male and female worms were mixed with equal amounts of proteins from each, only the Na,K-ATPase activity was significantly decreased \((F\) ratio = 56.61, \(P < 0.01\)) over a similar range of ivermectin concentrations. Since ivermectin exhibits concentration-dependent effects on both ATPases in female adult worms, this might provide an insight into other effects of the drug. However, the adjustment of the dose of ivermectin to obtain a nodular concentration of at least 40 ng/ml is therefore recommended in the complete chemotherapy of onchocerciasis.

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Keywords: Concentration-dependent effects; Ivermectin; Triphosphatase; Adult worms

1. Introduction

Ivermectin is a semi-synthetic macrocyclic lactone produced by the actinomycete, *Streptomyces avermitilis*. Although the long-term efficacy of a single dose is generally less than that of a full course of diethylcarbamazine (DEC), ivermectin has now replaced DEC as the treatment of choice for onchocerciasis (Coutinho et al., 1994). While DEC does not kill microfilariae in vitro, ultrastructural studies have shown that it exposes microfilariae to the body's defence mechanisms by acting on the cuticle of the parasite (Bryceonon et al., 1977). If ivermectin has a mechanism of action similar to that of DEC (Ette et al., 1990), then ivermectin may do more than just removing the cuticle of the worm since it has a sustained microfilaricidal effect. We are interested in those actions of ivermectin on the worm, before the involvement of the body's defence mechanisms. Moreover, ivermectin-facilitated immune responses may reach a critical importance only after several treatments with ivermectin (Soboslay et al., 1993), but we know that ivermectin has a...
microfilaricidal effect even after the first dose (Njoo et al., 1993).

Even though ivermectin has revolutionized the treatment of onchocerciasis, its effects on the adult worms have not been fully explored. There is some evidence in man that repeated courses may have a partial microfilaricidal or chemosterilant effect (Chavasse et al., 1992; Duke et al., 1992). This implies that adult worms of *Onchocerca volvulus* may be susceptible to doses of ivermectin greater than 150 μg/kg. A point of interest with these previous studies, which was probably overlooked, was the concentration of the drug in the plasma or nodules that caused such effects on the adult worms, bearing in mind that there is variable bio-availability in tissues (Okonkwo et al., 1994).

At a standard dose of 150 μg/kg, the mean maximum plasma concentration, $C_{max}$, is 38.2 ng/ml (Okonkwo et al., 1993). Recent studies (Shu et al., 1997) advocated the improvement of this dose to 300 μg/kg; yet the concentration that gets to the skin or nodules is not defined.

Considering that membrane-associated ATPases participate in a variety of cellular functions (Shu and Emeh, 1997), this study investigated the concentration of ivermectin that can have an inhibitory effect on the activities of ATPases in adult worms of *O. volvulus*. This is part of an ongoing study to adjust the dose of ivermectin administered to patients, to achieve an effective concentration on the adult worms.

2. Materials and methods

2.1. Subjects

Six male subjects aged between 20 and 40 years were involved in this study. They were recruited from an onchocerciasis-endemic region, Okpala in Ud当地 government area, Enugu, Nigeria. In a previous survey of the area, 58.3% of the population was skin-snip positive for onchocerciasis (Shu and Okonkwo, 1998). After careful clinical examination of the subjects, those with prominent palpable nodules were recruited. Further screening was done to exclude those who had taken ivermectin within the past 12 months, prior to the study. All subjects granted informed consent. This study received requisite approval from the Ethics Committee of the University of Nigeria Teaching Hospital, Enugu, Nigeria.

2.2. Nodulectomy and digestion of nodules

One nodulectomy was carried out on each subject, as previously described (Dallah et al., 1993). Xylocaine (4 ml; 1%) without adrenaline was used for local skin anaesthesia around the nodules. A skin incision (1–5 cm in length) was made over the nodule, which was identified and held by stay sutures. Dissection close to the nodule gave a bloodless field and delivered the nodule without difficulty. The wound was closed by the interrupted suture technique using silk. Oral paracetamol (3000 mg in divided doses, daily for 3 days) was offered as postoperative analgesic. The sutures were removed 1 week later.

The extirpated nodules were immediately immersed in normal saline, placed in a flask containing ice blocks and transported to the laboratory. While in the laboratory, the nodules were carefully trimmed with a pair of scissors to remove excess tissues. They were subjected to collagenase digestion by the method of Schulze-Key and Albiez (1977). The adult worms in the collagenase solution were identified and separated into two groups according to the sex of the worm.

2.3. Preparation of worms and drug

The female worms were transferred into Tyrode’s solution and left for 2 h at 4°C for microfilariae to migrate out of the worms. The microfilariae were then filtered out using a cheese cloth. Thereafter, male and female worms were homogenized separately. The worms were crushed in a pre-cooled ceramic mortar containing Tris-HCl buffer, pH 7.4, and using sand (treated in concentrated H2SO4) as an abrasive. The samples were filtered through cheese cloth and stored in aliquots at −20°C until required for enzyme assay.

Four stock solutions (2000, 4000, 5000, and 10000 ng/ml) of ivermectin were prepared.
2.4. Enzyme assay

Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as the standard. Prior to enzyme assays, two samples (from each subject) were considered: (1) homogenate from female worms (FW); and (2) a male and female worm homogenate (WM), mixed with equal amounts of proteins from each.

Total ATPase activity was assayed by a modified spectrophotometric method of Bonting (1970), as reported by Osbor et al. (1994). The incubation medium (pH 7.4) contained NaCl (100 mM), KCl (10 mM), MgCl₂ (0.25 mM), EDTA (0.1 mM), Tris-HCl (100 mM), ATP (25 mM), appropriate concentrations of ivermectin and enzyme extract (0.1 ml) in a reaction volume of 1 ml. The reaction was terminated with 0.4 ml 10% sodium dodecyl sulphate. Inorganic phosphate released was quantified by its reaction with ammonium molybdate as previously described (Fiske and Subbarrow, 1925).

The Mg-ATPase activity was assayed under identical conditions as the total but in the presence of ouabain (0.1 mM). Ouabain is a specific inhibitor of Na,K-ATPase whose activity was calculated as the difference between the total and Mg-ATPase activities.

ATPase activity was determined from a standard curve, using KH₂PO₄ as standard.

2.5. Data analysis

Data on the enzyme activities of worms treated with the different concentrations were subjected to one way analysis of variance (ANOVA). Significant differences between the means were compared using Duncan's New Multiple Range Test (Obi, 1986).

3. Results

Generally, the Mg-ATPase activity was significantly higher than that of Na,K-ATPase in both groups of worms, and the Mg-ATPase activity in male and female worms was 1.25 and 1.39 μmol/mg protein/h, respectively.

Table 1 summarizes the results of Mg- and Na,K-ATPase activities (μmol/mg protein/h) of female adult worms.

<table>
<thead>
<tr>
<th>Drug concentration (ng/ml)</th>
<th>Mg-ATPase</th>
<th>Na,K-ATPase</th>
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<tr>
<td>0</td>
<td>1.39*</td>
<td>1.37*</td>
</tr>
<tr>
<td>20</td>
<td>1.39*</td>
<td>1.24*</td>
</tr>
<tr>
<td>40</td>
<td>0.95*</td>
<td>0.62*</td>
</tr>
<tr>
<td>50</td>
<td>0.77*</td>
<td>0.48*</td>
</tr>
<tr>
<td>100</td>
<td>0.57*</td>
<td>0.12*</td>
</tr>
</tbody>
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* Mean enzyme activities with a common superscript are not significantly different (P>0.01).

Table 2 represents the enzyme activities (Mg- and Na,K-ATPases) of the worm mixture (WM).

<table>
<thead>
<tr>
<th>Drug concentration (ng/ml)</th>
<th>Mg-ATPase</th>
<th>Na,K-ATPase</th>
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<tr>
<td>9</td>
<td>2.64</td>
<td>1.56*</td>
</tr>
<tr>
<td>20</td>
<td>2.46</td>
<td>1.48*</td>
</tr>
<tr>
<td>40</td>
<td>2.46</td>
<td>0.98*</td>
</tr>
<tr>
<td>50</td>
<td>2.35</td>
<td>0.58*</td>
</tr>
<tr>
<td>100</td>
<td>2.34</td>
<td>0.08*</td>
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* Mean enzyme activities with a common superscript are not significantly different (P>0.01).
Fig. 1. Activities (%) of Mg- and Na,K-ATPase after a single dose of ivermectin.

the Mg-ATPase activity of the WM. However, a significant inhibitory effect ($F$ ratio = 56.61, $P < 0.01$) on the Na,K-ATPase activity was observed with similar increasing drug concentrations. Separation of means showed that ivermectin concentrations of 40 ng/ml and above significantly reduced ($P < 0.01$) the mean Na,K-ATPase activity of the WM. There was no significant difference ($P > 0.01$) between enzyme activities of worms treated with 40 and 50 ng/ml. However, a drug concentration of 100 ng/ml had a significant inhibitory effect on the mean enzyme activity of the WM when compared with drug concentrations of 0–50 ng/ml.

Fig. 1 represents the results of the percent activity of Mg- and Na,K-ATPase of WM at various concentrations of ivermectin, when compared with the controls. At 100 ng/ml, ivermectin had inhibitory effects of 11.36 and 94.87% on Mg- and Na,K-ATPase activities, respectively.

4. Discussion

Studies on the mechanism of action of the avermectins have relied on work with the free living nematodes, Caenorhabditis elegans and the parasitic nematode, Ascaris suum. Earlier studies suggested the modulation of GABA-mediated neurotransmission (Eine et al., 1990). More recently, the most likely explanation for how ivermectin works is that it specifically increases membrane chloride ion permeability (Turner and Schaeffer, 1989). It has been reported that ivermectin also facilitates cellular immunity in treated patients (Soboslay et al., 1993). However, there may be other sites of action at which ivermectin affects target organisms (Turner and Schaeffer, 1989). This study advances one of such sites.

In this study, mean Na,K-ATPase showed a marked decrease ($P < 0.01$) in specific activity with increase concentration of ivermectin, in both FWs and WM. These results point to a relationship between the drug and these membrane ATPases.

Unlike the FWs, there was no significant decrease ($P > 0.01$) in mean activity of Mg-ATPase in the WM. Since equal amounts of proteins from male and female worms were used, and the activity of Mg-ATPase in the FW was affected by the drug, one would have expected that enzyme activity in the WM would also have been affected. That was not the case. Moreover, since the specific activity of the FW (1.39 pmol/mg protein/h) was higher than that of the males (1.25 pmol/mg protein/h), it did not mean that the effect of the drug was not to be felt by the WM. In a previous study (Duke et al., 1992), male worms were suspected to have migrated away from the nodules leaving the females, after treatment with high doses of ivermectin. While this supports the view that ivermectin affects male and female worms differently, it does not explain why Mg-ATPase in the WM did not change significantly in this study. The small inhibition observed in the WM was probably the contribution of the inhibition from the female worms. However, this differential effect of ivermectin on Mg-ATPase activity may form the basis for further research work.

From the results of this study, one may be tempted to say that ivermectin, at doses higher than those previously administered (Chavasse et al., 1992; Duke et al., 1992) can cause the death of female macrofilariae of *O. volvulus*, considering the importance of ATPases in metabolism. Thus, the inhibition of monovalent cation (Na +,K+) and divalent cation (Mg2+,Ca2+) transport across the membrane through Na,K and Mg pumps will have a great physiological and biolog-
ical effect on the worms. Owing to these essential functions of ATPases (nerve transmission, coordination, metabolism, motility, respiratory system and organ functions in general), it is possible that the death of these worms may partially result from the inhibition of these enzymes. Since filarial metabolism would be radically affected, the production and/or secretion of immunomodulatory factors (Eikhalifa et al., 1991), which enable filariae to circulate in the human body without eliciting an apparent host immune response, might be disturbed. Subsequently, it is likely that filarial defense mechanisms may be inhibited, as the ATPase enzymes are altered, leading to the recognition and destruction by the host immune system.

Considering the inhibitory effects of ivermectin on Mg- and Na,K-ATPase activities in female adult worms, it is suggested that the dose administered to patients be adjusted to obtain a nodular concentration of at least 40 ng/ml in the nodule. If that is done, much would have been achieved in the complete chemotherapy of onchocerciasis using ivermectin.

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