

CHARACTERIZATION OF THE HEAT-RELEASED SUBSTANCE FROM RAT ILEAL MUSCLE

Zoheir A. Damanhour, Osama S. Tayeb

Department of Pharmacology, Faculty of Medicine and Allied Sciences, and Heat Stroke Research Unit, King Fahad Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia

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Abstract: Previous studies have proposed that increasing the temperature around the rat isolated ileal muscles induces a heat-released substance (HRS) which causes a decrease in the contractile response. Therefore, we have studied various characteristics of this proposed HRS in animals exposed to heat-stress conditions. In these animals, the contraction of muscles in response to carbachol was decreased by increasing the temperature from 37°C to 40°C. Further, bathing the ileum in a conditioned medium prepared by incubation of the ileal muscle at 40°C has shown less contraction at 40°C than in presence of normal Krebs medium. In addition, conditioned mediums, prepared at 40°C were run on SDS-polyacrylamide gel electrophoresis and have shown the presence of a distinct protein band at a m.w. region of 55 kDa. These results confirm our previous studies that increasing the temperature around the muscles induces the HRS that causes the decrease in the contractile response.

Address for correspondence: Professor Osama S. Tayeb, Department of Pharmacology, Faculty of Medicine & Allied Sciences, King Abdulaziz University, P.O. Box 9029, Jeddah 21413, Saudi Arabia.

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Overexposure to heat causes several changes in many physiological functions in the body. Among these are central nervous system manifestations [1, 12], liver damage [9], and acute renal failure [15]. A wide range of biochemical changes have also been observed in heat stroke victims [2, 3, 4]. Various mechanisms were suggested for these changes [13, 14, 15]. Among these we have proposed a release of a heat-released substance (HRS) from ileal muscles [6] and from diaphragmic muscles [7]. The evidence for the presence of this mediator was based on pharmacological interpretations, but nothing is known about its composition or identity. Therefore, we have studied various characteristics of this HRS in order to delineate its actions and identity.

MATERIALS AND METHODS

Isolated rat ileum preparations. The effect of carbachol on isolated rat ileum was examined in the same

manner as described previously [5]. After dissecting the rat, short segments from the caecal end of the ileum were removed and put immediately into a petri dish containing oxygenated (95% O₂/5% CO₂) Krebs solution of the following composition – NaCl: 6.9 g/l; Na HCO₃: 2.1 g/l; glucose: 2 g/l; KH₂PO₄: 0.16 g/l; KCl: 0.36 g/l; MgSO₄.7H₂O: 0.29 g/l; CaCl₂.2H₂O: 0.37 g/l. Isolated strips of muscles were trimmed, and one of these strips was transferred to a 10 ml muscle bath at 37°C. The muscle was washed with Krebs solution and left for 30 minutes for equilibration. Muscle contractions were recorded by a Palmer Bioscience potentiometric recorder connected to the muscle through a Bioscience UF1 isometric transducer and A100 Bioscience coupler. Carbachol was added to the muscle bath in various concentrations in a graded manner. The muscle was washed after each addition of carbachol. This was continued until the dose that caused a maximum contraction of the muscle was reached. Then, in some

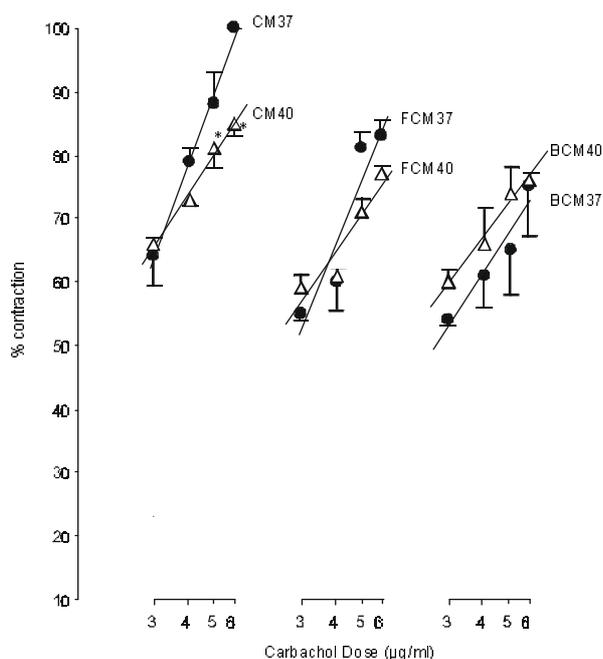


Figure 1. Log dose-response curves of the effect of carbachol on the contraction of the isolated rat ileum at 37°C in the presence of CM, BCM, or FCM. Each point is the average of at least 6 experiments. Lines above and below points represent SEM. CM represents conditioned medium prepared at either 37°C (CM37) or 40°C (CM40). BCM was prepared by incubating the prepared conditioned medium at either 37°C (BCM37) or 40°C (BCM40) in a boiling water bath for 60 minutes. FCM was prepared by freezing the prepared conditioned medium at either 37°C (FCM37) or 40°C (FCM40) at -4°C overnight. * $p < 0.01$ indicates a significant difference versus the responses in the presence of CM37.

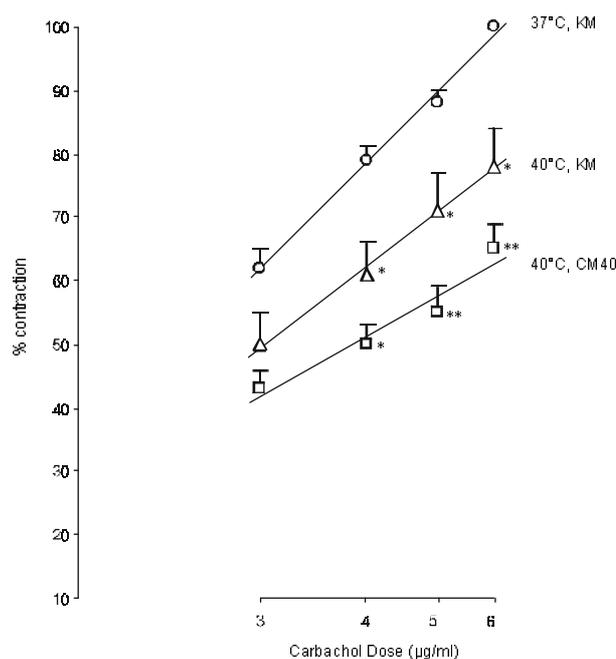


Figure 2. Log dose-response curves of the effect of carbachol on the contraction of the isolated rat ileum in the following conditions: at 37°C, and at 40°C in the presence or absence of CM40. Each point is the average of at least 6 experiments. Lines above and below points represent SEM. KM represents freshly prepared Krebs medium around the muscle. CM40 represents a conditioned medium prepared by incubating the ileal muscle at 40°C for 30 minutes. The temperature shown beside the curve represents the muscle bath temperature. * $p < 0.01$, ** $p < 0.001$ indicate significant difference versus the responses at 37°C, KM.

experiments, bath temperature was changed to 40°C and left for equilibration for 30 minutes, before the same series of carbachol doses was again tested. Variation in exposure conditions and preparations of conditioned mediums were performed as previously described [7].

Preparation of conditioned mediums. Conditioned mediums (CM) were prepared by incubating a piece of ileal muscle (similar in size to the muscle used in the muscle bath) in 10 ml Krebs solution, oxygenated by 95% O₂/5% CO₂, at either 37°C (CM37) or 40°C (CM40) for 30 minutes. Boiled conditioned medium was prepared by incubating the prepared CM for 60 minutes in a boiling water bath (BCM). Frozen conditioned medium was prepared by freezing the prepared CM at -4°C overnight (FCM). Freshly prepared Krebs medium was referred to as (KM). In some experiments conditioned medium was prepared using ileal muscles from heat-stressed animal model (HCM).

Effect of boiled and frozen conditioned mediums. Responses of the ileal muscle to various doses of carbachol were tested in some experiments in the presence of KM, BCM, or FCM mediums. The temperature of

these mediums were readjusted to 37°C before being used to bathe the ileal muscle.

Actions of carbachol on isolated ileal muscles from heat exposed animals. In some experiments, the effects of various doses of carbachol were tested on ileal muscles obtained from animals which had been exposed to high ambient temperature in the same manner as described previously [6]. Animals were exposed to heat stress by placing them, restrained in a cage, within an environmental room (Hotpack, Philadelphia, USA) controlled at 40°C. Rectal temperature was monitored every five minutes throughout the experiment by means of a rectal probe (3.5 cm insertion). The probe was connected to an electronically controlled temperature measuring device through copper constantan thermocouples (Isothermix, Columbus Instruments, Ohio, USA). Exposure of test animals to the above-mentioned stress situations was continued for about an hour until a rectal temperature of 40.5°C had been maintained for 10–15 minutes. At this point, the animals were considered heat-stressed. All animals were allowed access *ad lib* to standard laboratory diets and tap water, but fasted overnight before the heat stress challenge. During the stress periods all animals

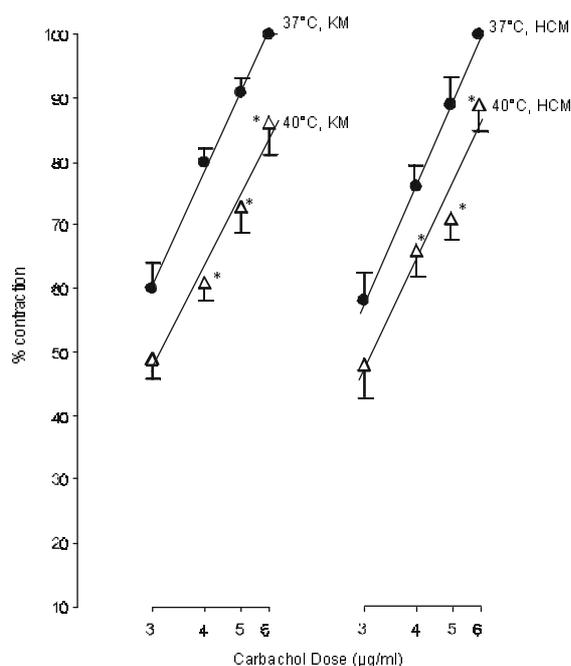


Figure 3. Log dose-response curves of the effect of carbachol on the contraction of the isolated rat ileum from control rats (left) and heat-stressed rats (right). Each point is the average of at least 6 experiments. Lines above and below points represent SEM. KM represents freshly prepared Krebs medium around the muscle from control rats. HCM represents a conditioned medium prepared using ileal muscles from heat-stressed rat. The temperature shown beside the curves represents the muscle bath temperature. * $p < 0.05$ indicates significant difference versus the correspondent response at 37°C.

were watched closely for any signs of exhaustion, over stress, or increased rectal temperature (above 40.5°C). Experiments were discontinued immediately if any of the above signs were observed. These exposure conditions were established in such a way to have a minimal mortality rate of animals (<10%) during and after heat stress.

The use of this animal model for heat stress studies was approved by the Ethics Committee of King Fahad Medical Research Center in our Institution.

Determination of electrolytes, glucose and protein concentration. Concentration of electrolytes and glucose in Krebs solution and conditioned mediums was determined using an automated analyzer (Hitachi 705, Boehringer, Ingelheim, Germany). Protein concentrations were determined by the Lowry method [11].

Gel electrophoresis. Samples of the conditioned mediums prepared at 37°C and 40°C from control and heat-stressed rats were concentrated and run on electrophoresis gel for proteins separation in the same manner as described by Laemmli [10]. This experiment was repeated six times using six different samples.

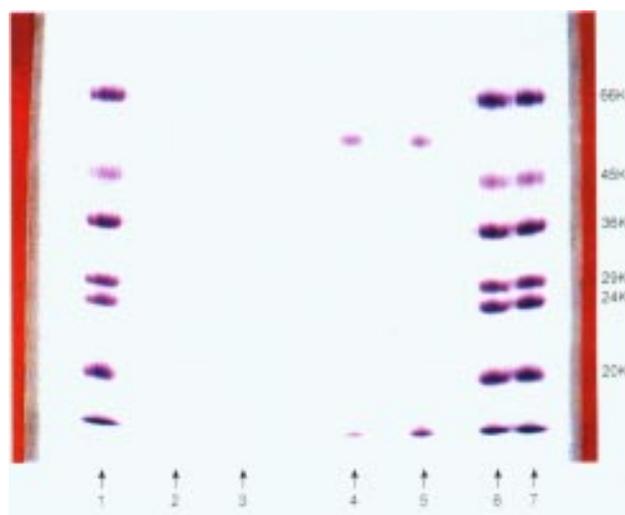


Figure 4. SDS-polyacrylamide gel electrophoresis of different conditioned mediums. Migration was from top to bottom. Staining was performed using Coomassie Brilliant Blue. 1, 6 and 7 are molecular weight standards; 2 and 3 are CM37; 4 and 5 are HCM40. CM37 represents a conditioned medium from control rats prepared at 37°C. HCM40 represents conditioned medium prepared at 40°C using ileal muscles from heat-stressed rats.

Statistical analysis. Each experiment was repeated at least six times; results were averaged and compared to the corresponding control using Student's t-test.

RESULTS

Effect of different conditioned mediums on ileal muscle incubated at 37°C. Figure 1 shows the dose-response curves of the effect of carbachol on isolated rat ileal muscle at 37°C in the presence of CM37, CM40, BCM37, BCM40, FCM37 and FCM40. In general, the responses to carbachol in the presence of BCM37 and FCM37 mediums were lower than the responses in the presence of CM37 medium. Comparison of the responses in the presence of BCM37 to the responses in BCM40 shows no difference in the response. Similarly, the responses in the presence of FCM37 did not differ from the responses in FCM40 medium. On the other hand, CM40 showed lower response to carbachol than responses observed in the presence of CM37 ($p < 0.01$).

Effect of carbachol on ileal muscle incubated at 40°C. Figure 2 shows the dose response curve of the effect of carbachol at 40°C in the presence and absence of CM40 as compared to its effect at 37°C. The results show that the effects of carbachol are lower at 40°C compared with its effect at 37°C. Further, the effect of carbachol at 40°C in the presence of CM40 is lower than the effect at 40°C in the presence of normal Krebs medium.

Responses of ileal muscle from heat exposed rats to carbachol. Figure 3 shows the dose-response curves of the effect of carbachol at different bath temperature or mediums on isolated ileal muscles from control and heat-

Table 1. Chemical composition of Krebs medium before and after conditioning at various temperatures.

Substance	Concentrations (mMol/l)			
	KM	CM30	CM37	CM40
Na ⁺	148 ± 3	151 ± 1	152 ± 1	151 ± 1
K ⁺	6.2 ± 0.3	6.5 ± 0.1	6.7 ± 0.1	7 ± 0.2
Mg ⁺⁺	0.6 ± 0.06	0.7 ± 0.06	0.7 ± 0.05	0.8 ± 0.1
Ca ⁺⁺	2.3 ± 0.1	2.7 ± 0.1 ^b	2.7 ± 0.1 ^b	2.6 ± 0.1 ^b
PO ₄ ⁻⁻⁻	1.1 ± 0.1	1.5 ± 0.1 ^b	1.5 ± 0.1 ^b	1.6 ± 0.1 ^b
Cl ⁻	135 ± 1	135 ± 2	135 ± 2	135 ± 2
Glucose	11 ± 1	9.2 ± 1.0 ^b	8.7 ± 1.3 ^b	8.7 ± 1.1 ^b
Proteins ^a	0.0 ± 0.0	139 ± 12 ^b	142 ± 24 ^b	198 ± 18 ^{b, c}

Figures represent means ± SEM of 8 experiments: ^aProtein concentration is expressed in µg/ml; ^bSignificant difference as compared to KM ($p < 0.01$); ^cSignificant difference as compared to CM30 and CM37 ($p < 0.01$). KM: Krebs medium; CM30: Conditioned medium prepared at 30°C; CM37: Conditioned medium prepared at 37°C; CM40: Conditioned medium prepared at 40°C.

stressed rats. It was quite obvious that the actions of carbachol on these isolated muscles from heat-stressed rats were affected in a similar way as reported on isolated ileal muscles from normal rats, i.e. the effects of carbachol were less potent at 40°C in HCM medium than carbachol effects at 37°C in HCM medium ($p < 0.05$).

Electrolytes, proteins and glucose contents of some conditioned mediums. Table 1 shows the electrolytes, proteins and glucose contents of conditioned mediums CM30, CM37 and CM40 as compared to normal Krebs medium. It is obvious that in all types of CM's there was an increase in the concentration of calcium ($p < 0.01$), phosphate ($p < 0.01$), and proteins ($p < 0.01$), while there was a decrease in the concentration of glucose ($p < 0.01$). Comparison of conditioned mediums one to another shows that the protein contents in CM40 is significantly higher than its concentration in CM30 or CM37 ($p < 0.01$).

Proteins electrophoresis. Figure 4 shows a sample result of conditioned mediums electrophoresis from control and heat-stressed rats. The result shows the presence of a dense band of protein at a m.w. of ~ 55,000 Da in the HCM40 sample. This band was not observed in the CM37 sample from control rats. This finding was confirmed in the other five repetitions performed.

DISCUSSION

The decrease in the effect of carbachol on the contraction of isolated rat ileum incubated at 40°C, as compared to the effect if incubated at 37°C, was reported previously [5]. In the present investigation we have reported a similar action on ileal muscles isolated from animals exposed to heat-stress conditions. This confirms

our previous suggestion of the release of a substance that causes a decrease in the contractile response of smooth muscles. It may also indicate that there is no acclimatization or tolerance developed to the effect of this substance, as the effect was observed even in muscles isolated from animals exposed to high temperature.

Further, we have shown in this investigation that this heat released substance can cause an additional decrease in the response of the ileal muscle incubated at 40°C. This would further confirm our finding of the release of a heat-released substance from ileal muscles upon exposure to surrounding high temperature [7], and it may indicate that the effect of this heat-released substance is quantitative in nature, i.e. dose related. Several mechanisms could cause this type of response. More studies from this aspect are being carried out in our laboratory.

The chemical analysis of the composition of the various types of the conditioned mediums showed a striking finding, namely the presence of a higher amount of proteins in CM40 as compared to CM37 or CM30 (Tab. 1). It also showed that both CM 37 and CM 40 mediums have similar significant differences in some of the electrolytes (i.e. Ca⁺⁺, PO₄⁻⁻⁻) and glucose compared to the KM control medium. Therefore, changes in carbachol dose response in the presence of CM40 is most likely due to the increased amount of protein, and not to changes in electrolytes or glucose. This would indicate that upon incubation of ileal muscles at 40°C an extra amount or type of protein is released. This may be the cause of the decrease in the contractile responses of ileal muscles incubated at 40°C, i.e. it may be the HRS as was postulated previously [7]. This is further confirmed by the observed abolished effect of CM40 after boiling or freezing. It is well known that boiling and/or freezing deactivate the biological activities of proteins [8].

In conclusion, heating the ileum *in vitro* in Krebs solution at 40°C in contrast to 37°C, inhibits the response by releasing HRS. A further inhibition is obtained by using CM40, which possibly contains HRS. The fact that similar results were obtained when ileum was used from heat-stressed rats, indicates no development of tolerance to the releasing effect of HRS. Further, the change in chemical composition (except for putative HRS protein) of the conditioned medium CM40 is not responsible for the inhibition of the response.

The presence of a novel protein released upon incubation of ileal muscles at 40°C is strikingly shown by our preliminary work on gel electrophoresis of CM's which shows a dense protein band in the molecular weight range of 55 kDa. This may well be the HRS proposed. More investigations are being carried out in this aspect in our laboratory.

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