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18β-甘草次酸钠对大鼠嗜中性白细胞活性氧生成及细胞内游离 Ca²⁺浓度的影响

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Effects of sodium 18β-glycyrrhetic acid on the production of active oxygen species and the concentration of intracellular free Ca²⁺ in rat neutrophils

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ABSTRACT The effects of sodium 18β-glycyrrhetic acid (SGA) on the production of active oxygen species (AOS) and the concentration of intracellular free Ca²⁺ ([Ca²⁺]_i) in the rat neutrophils were studied by the methods of luminoldependent chemiluminescence and calcium indicator Fura-2 loading. The results showed that SGA 0.8-12.7 μmol · L⁻¹ selectively inhibited calcium inophore A-23187-stimulated AOS production with IC₅₀ 1.4 μmol · L⁻¹; while SGA 25.4-203 μmol · L⁻¹ stimulated neutrophils to generate AOS and potentiated the action of chemotactic peptide f-MLP on inducing AOS production. SGA 25.4-203 or 6.3-50.7 μmol · L⁻¹ depressed the [Ca²⁺]_i rise in neutrophils stimulated by A-23187 or f-MLP; in the contrast, verapamil or diltiazem has higher effective concentration more than 100 or 200 μmol · L⁻¹ to f-MLP and left [Ca²⁺]_i rise induced by A-23187 unaffected. SGA 101.5-203 μmol · L⁻¹ also caused [Ca²⁺]_i rise in

neutrophils. The result analysis indicate that the effect of SGA on AOS production has no relationship with its regulation to [Ca²⁺]_i in neutrophils.

KEY WORDS glycyrrhetic acid; neutrophils; oxygen; calcium

摘要 18β-甘草次酸钠(SGA) 0.8-12.7 μmol · L⁻¹ 选择性地抑制 A-23187 诱导的 Neu 活性氧生成; 25.4-203 μmol · L⁻¹ 刺激 Neu 生成活性氧, 并增强 f-MLP 的活性氧生成作用. SGA 25.4-203 或 6.3-50.7 μmol · L⁻¹ 抑制 A-23187 或 f-MLP 诱导的 Neu [Ca²⁺]_i 增加, 101.5 μmol · L⁻¹ 以上也有升高 Neu[Ca²⁺]_i 的作用.

关键词 甘草次酸; 嗜中性白细胞; 氧; 钙

18β-甘草次酸钠(sodium 18β-glycyrrhetic acid, SGA)是甘草甜素水解产物甘草次酸(glycyrrhetic acid)光学异构体的钠盐, 有抗炎、抑制炎症组织前列腺素 PGE₂ 生成和拮抗炎症介质活性的作用^(1,2). 近年的研究表明, 白细胞在受刺激时释放的活性氧也和急性炎症密切相关^(3,4). 为进一步阐明 SGA 的作用机制, 本文探讨了其对大鼠嗜中性白细胞(Neu)活性氧生成及对 Neu 细胞内游离 Ca²⁺浓度[Ca²⁺]_i 的影响.

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MATERIALS AND METHODS

f-MLP (formyl-methionyl-L-leucyl-L-phenylalanine)和钙离子载体 A-23187 为 Sigma 产品, 溶在适量 DMSO (dimethyl sulfoxide)中, 用蒸馏水稀释. luminol (3-aminophthaloyl hydrazine, 和光纯药) 10 mg, 加 40 μl 三乙胺后用磷酸缓冲盐水 (PBS, pH 7.4)稀释至 2 $\text{mg} \cdot \text{ml}^{-1}$. 吲哚美辛 (indomethacin)、维拉帕米 (verapamil)、硫氮萘酮 (diltiazem)和 3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazoline (BW 755-C) 为 Sigma 产品; SGA 由宁夏化工研究所提供, 纯度 95-98%, 均溶在 50% DMSO 中. Eagle's MEM 培养基(日水制药)用蒸馏水溶解 (9.4 $\text{g} \cdot \text{L}^{-1}$, Ca^{2+} 1.8 $\text{mmol} \cdot \text{L}^{-1}$), 用饱和 NaHCO_3 溶液调至 pH 7.0.

调理化酵母聚糖 A (opsonized zymosan A) 的制备 酵母聚糖 A (Sigma) 50 mg 加 1 ml 新鲜大鼠血清后在 37 $^{\circ}\text{C}$ 孵 30 min, 3000 \times g, 4 $^{\circ}\text{C}$ 离心 10 min. 沉淀用 PBS 洗两次后混悬在 1 ml PBS 中, -80 $^{\circ}\text{C}$ 保存.

Neu 悬液的制备 Wistar 大鼠, 雌雄不拘, 体重 230 \pm SD 15 g, ip 液体石蜡 10 ml. 18 h 时放血处死. 用冰中预冷的 MEM 60 ml 分两次洗出腹腔渗出液, 70 \times g, 4 $^{\circ}\text{C}$ 离心 5 min. Neu 沉淀再用 MEM 洗一次后悬浮在 MEM 中. 冰中稳定 1 h 后进行化学发光测定.

化学发光测定 Neu 悬液 500 μl 与 luminol 10 μl (终浓度 40 $\mu\text{g} \cdot \text{ml}^{-1}$)混合后加 2.5-5 μl 药液或等容积 DMSO (终浓度 <2.5 $\mu\text{l} \cdot \text{ml}^{-1}$), 37 $^{\circ}\text{C}$ 温育 10 min; 加 10 μl 刺激剂 (f-MLP, A-23187 或调理化酵母聚糖 A) 进行刺激. 用 Biolumat LB 9500-SII 6 频道化学发光分析器连续进行光子计数 ($\lambda=460$ nm). 用化学发光最大值作为测定指标, 计算药物的抑制率(%), 测定时间 10-30 min.

Fura-2 标记 Neu 的制备 上述大鼠腹腔

渗出液 70 \times g, 4 $^{\circ}\text{C}$ 离心 10 min, 沉淀用 Fura-2-AM (和光纯药) 4 $\mu\text{mol} \cdot \text{L}^{-1}$ 的 MEM 悬浮, 37 $^{\circ}\text{C}$ 温育 30 min, 用 MEM 洗两次后制成 1×10^7 细胞/ml 的 Neu-MEM 悬浮液.

Neu $[\text{Ca}^{2+}]_i$ 的测定 250 μl Fura-2 标记 Neu 悬液加 2.5-5 μl 药液或等容积 DMSO (<5 $\mu\text{l} \cdot \text{ml}^{-1}$), 37 $^{\circ}\text{C}$ 温育 5 min. 加 10 μl 刺激剂 (f-MLP 或 A-23187) 诱发 $[\text{Ca}^{2+}]_i$ 增加, 用 FP-770 荧光分光光度计在 37 $^{\circ}\text{C}$ 、搅拌的条件下连续测定荧光增加值 (激发波长 335 nm、狭缝 10 nm, 发射波长 500 nm、狭缝 10 nm).

全部实验重复 4 次. 药物的 IC_{50} (95% 可信限)用对数浓度对抑制率经直线回归⁽⁵⁾后求得.

RESULTS

SGA 刺激 Neu 生成活性氧的作用 Fig 1 A 和 Tab 1 结果表明, SGA 25.4-203 $\mu\text{mol} \cdot \text{L}^{-1}$ 刺激 Neu 生成活性氧. SGA 为 25.4, 50.7, 101.5 和 203 $\mu\text{mol} \cdot \text{L}^{-1}$ 时, 活性氧生成的潜伏期分别为 23.0 ± 0.6 , 18.9 ± 1.2 , 15.0 ± 0.8 和 15.0 ± 0.6 s, 较 f-MLP 0.5 $\mu\text{mol} \cdot \text{L}^{-1}$ 潜伏期 (5.6 ± 0.5 s) 明显为长, 较 A-23187 5 $\mu\text{mol} \cdot \text{L}^{-1}$ 潜伏期 (34.1 ± 2.7 s) 稍短. SGA 刺激的活性氧生成在 1.5 min 左右达高峰. 3-7 min 恢复正常. 作用在 101.5 $\mu\text{mol} \cdot \text{L}^{-1}$ 时达最大, log 浓度-效应曲线的直线部分经直线回归⁽⁵⁾后求得 EC_{50} 为 40.4 $\mu\text{mol} \cdot \text{L}^{-1}$, $r=0.9725$ ($P<0.01$). 12.7 $\mu\text{mol} \cdot \text{L}^{-1}$ 以下 SGA 无活性氧生成刺激作用.

SGA 对 f-MLP 活性氧生成作用的增强 SGA 与 Neu 共孵 10 min (SGA 的活性氧生成刺激作用已消失), 加入 f-MLP (0.5 $\mu\text{mol} \cdot \text{L}^{-1}$). 结果 (Tab 1 和 Fig 1B) 加与不加 SGA 组比较, f-MLP 诱导的化学发光计数明显增加, 说明预加入 SGA 增强 f-MLP 刺激

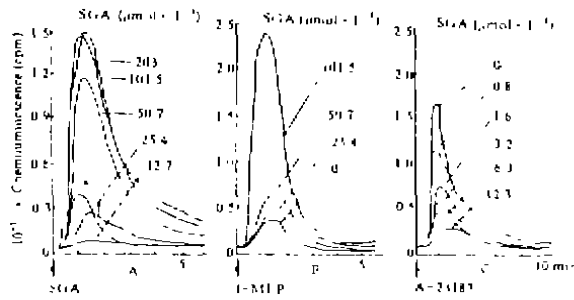


Fig 1. The stimulatory action of sodium 18β-glycyrrhetic acid (SGA) on the production of active oxygen species (A) and the effect of SGA on the production of active oxygen species induced by f-MLP (B) or A-23187 (C). Neutrophils: 1×10^7 cells \cdot ml $^{-1}$. * Only stimulated by f-MLP 0.5 μ mol \cdot L $^{-1}$. In B and C, SGA was added 10 min before challenged by f-MLP (0.5 μ mol \cdot L $^{-1}$) or A-23187 (5 μ mol \cdot L $^{-1}$).

Neu 生成活性氧的作用。BW 755-C (0.2–200 μ mol \cdot L $^{-1}$)和吲哚美辛(20–400 μ mol \cdot L $^{-1}$)则呈明显抑制作用, IC₅₀ (95%可信限)分别为 1.9 (1.3–2.9)和 68.4 (34.0–137.4) μ mol \cdot L $^{-1}$ 。

SGA 对调理化酵母聚糖 A 活性氧生成作

Tab 1. Stimulatory effect of sodium 18β-glycyrrhetic acid (SGA) on production of active oxygen species of rat neutrophils and interaction with f-MLP. Neutrophils: 1×10^7 cells \cdot ml $^{-1}$. $n=4$, $\bar{x} \pm$ SD. *** $P < 0.01$ vs f-MLP alone.

SGA (μ mol \cdot L $^{-1}$)	f-MLP (μ mol \cdot L $^{-1}$)	Chemiluminescence (cpm)	Ratio of effect
0	0.5	32 246 \pm 2 614	1.00
12.7	0	2 415 \pm 518	0.07
25.4	0	22 345 \pm 3 571	0.69
50.7	0	100 834 \pm 24 4157	3.13
101.5	0	142 948 \pm 30 948	4.43
203	0	137 318 \pm 19 724	4.25
0	0.5	31 217 \pm 343	1.00
25.4	0.5	37 843 \pm 3 693***	1.21
50.7	0.5	57 816 \pm 4 182***	1.85
101.5	0.5	223 437 \pm 16 723***	7.16

用的影响 SGA 12.7 μ mol \cdot L $^{-1}$ 与 Neu 共孵 10 min 后, 加入调理化酵母聚糖 A (0.5 mg \cdot ml $^{-1}$), 结果溶媒对照组和 SGA 组化学发光计数分别为 523 917 \pm 25 734 和 521 146 \pm 24 819 cpm, 无显著性差别($P > 0.05$), 说明 SGA 对

Tab 2. Inhibitory effect of sodium 18β-glycyrrhetic acid (SGA) on production of active oxygen species of rat neutrophils stimulated by calcium ionophore A-23187 (5 μ mol \cdot L $^{-1}$). Neutrophils: 1×10^7 cells \cdot ml $^{-1}$. $n=4$, $\bar{x} \pm$ SD. *** $P < 0.01$ vs control.

Drugs	Concn (μ mol \cdot L $^{-1}$)	Chemiluminescence (cpm)	Inhibition (%)	IC ₅₀ (95%) confidence limits, μ mol \cdot L $^{-1}$)
Control	—	173 256 \pm 5 645	—	
SGA	0.8	100 489 \pm 10 828***	42.0 \pm 6.3	1.4(1.1–1.7)
	1.6	81 690 \pm 7 643***	52.9 \pm 4.4	
	3.2	67 245 \pm 7 173***	61.2 \pm 4.1	
	6.3	50 942 \pm 6 809***	70.6 \pm 3.9	
	12.7	34 604 \pm 12 543***	80.0 \pm 7.2	
	25.4	19 926 \pm 9 581***	88.5 \pm 5.5	
BW 755-C	0.2	138 553 \pm 5968***	20.0 \pm 3.4	2.2(1.5–3.2)
	2	108 267 \pm 10 326***	37.5 \pm 6.0	
	10	55 051 \pm 13 323***	68.2 \pm 7.7	
	20	12 093 \pm 6 989***	93.0 \pm 4.0	
Indomethacin	100	119 321 \pm 7 415**	31.1 \pm 4.3	211.9(179.3–250.3)
	200	96 293 \pm 8 732***	44.4 \pm 5.0	
	400	52 480 \pm 14 293***	69.7 \pm 8.3	

酵母多糖 A 诱导的活性氧生成无抑制作用。BW 755-C 10-100 $\mu\text{mol} \cdot \text{L}^{-1}$ 抑制酵母多糖 A 诱导的活性氧生成, IC_{50} (95%可信限)为 13.1 (10.4-16.5) $\mu\text{mol} \cdot \text{L}^{-1}$ 。

SGA 对 A-23187 活性氧生成作用的抑制 SGA 与 Neu 共孵 10 min 后, 加入 A-23187 5 $\mu\text{mol} \cdot \text{L}^{-1}$ 。Tab 2 和 Fig 1 C 显示 SGA 0.8-25.4 $\mu\text{mol} \cdot \text{L}^{-1}$ 对 A-23187 诱导的 Neu 活性氧生成呈浓度依赖性的抑制, 其抑制强度较 BW 755-C 略强。SGA 12.7 和 25.4 $\mu\text{mol} \cdot \text{L}^{-1}$ 使 A-23187 诱导活性氧生成的潜伏期由对照的 $37.9 \pm 0.6 \text{ s}$ 分别延长至 44.2 ± 1.5 和 $65.7 \pm 2.3 \text{ s}$ (P 分别 < 0.01)。

SGA 诱导 Neu $[\text{Ca}^{2+}]_i$ 增加的作用 SGA 无荧光产生和淬灭作用。Fura-2 标记 Neu 一起温育 5 min 时加入 SGA, 即时出现荧光增加 (Fig 2 C), 曲线与 A-23187 的极为相似, 持续 10 min 以上而不衰减。和 A-23187 同样无潜伏期, 有效刺激浓度在 101.5 $\mu\text{mol} \cdot \text{L}^{-1}$ 以上。Fig 3 显示 SGA 诱导 $[\text{Ca}^{2+}]_i$ 增加和活性氧生成的量-效曲线大致平行。

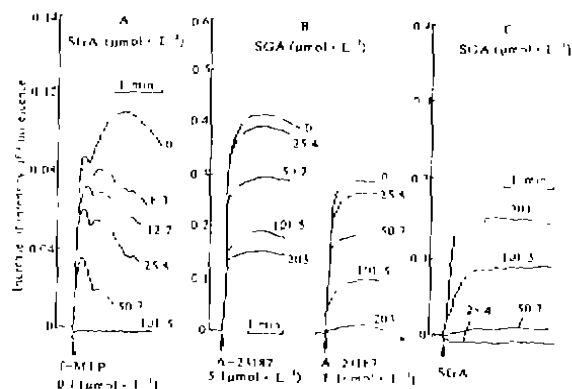


Fig 2. The stimulatory action of sodium 18 β -glycyrrhetic acid (SGA) on the rise of $[\text{Ca}^{2+}]_i$ in neutrophils (C) and the inhibitory effect of SGA on the rise of $[\text{Ca}^{2+}]_i$ in neutrophils stimulated by f-MLP (A) or A-23187 (B). Neutrophils: $1 \times 10^7 \text{ cells} \cdot \text{ml}^{-1}$. In A and B, SGA was added 5 min before challenged by f-MLP or A-23187.

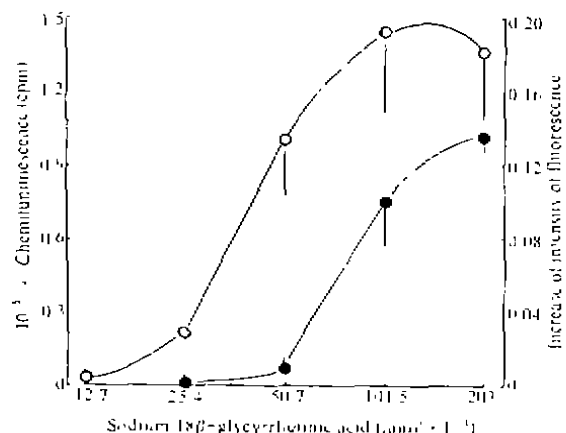


Fig 3. The relationship between production of active oxygen species and $[\text{Ca}^{2+}]_i$ rise in neutrophils stimulated by sodium 18 β -glycyrrhetic acid. (○): chemiluminescence, (●): increase of intensity of fluorescence. $n=4$, $\bar{x} \pm \text{SD}$.

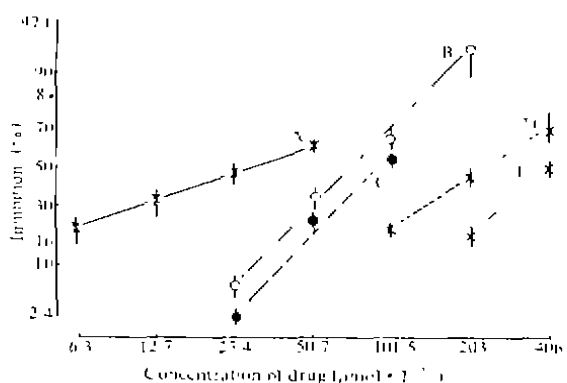


Fig 4. The inhibitory effect of sodium 18 β -glycyrrhetic acid (SGA) on the increase of intensity of intracellular fluorescence of Fura-2 loaded neutrophils stimulated by f-MLP 0.1 $\mu\text{mol} \cdot \text{L}^{-1}$ (×) or A-23187 1 (○) and 5 (●) $\mu\text{mol} \cdot \text{L}^{-1}$. The increase of intensity of fluorescence of control: 0.108 ± 0.016 by f-MLP, 0.423 ± 0.015 by A-23187 5 $\mu\text{mol} \cdot \text{L}^{-1}$ and 0.284 ± 0.013 by A-23187 1 $\mu\text{mol} \cdot \text{L}^{-1}$. The IC_{50} values of SGA (A, B and C): 29.2, 71.4 and 96.2, respectively; of verapamil (D): 225.7; of diltiazem (E): 398.3 $\mu\text{mol} \cdot \text{L}^{-1}$. $n=4$, $\bar{x} \pm \text{SD}$.

SGA 对 A-23187 促 $[\text{Ca}^{2+}]_i$ 增加作用的抑制 SGA 25.4-203 $\mu\text{mol} \cdot \text{L}^{-1}$ 明显抑制 A-23187 刺激的 $[\text{Ca}^{2+}]_i$ 增加 (Fig 2 B, Fig 4)。A-23187 1 或 5 $\mu\text{mol} \cdot \text{L}^{-1}$ 时, 最高抑制率分别为 95.8 和 62.6%。Verapamil 和 diltiazem

均无抑制作用。

SGA 对 f-MLP 促[Ca²⁺]_i 增加作用的抑制 SGA 6.3 - 50.7 μmol · L⁻¹ 明显抑制 f-MLP 诱导的 Neu [Ca²⁺]_i 增加, 使 f-MLP 的双相[Ca²⁺]_i 增加曲线明显降低(Fig 2 A), 较 diltiazem 和 verapamil 的作用明显为强(Fig 4)。

DISCUSSION

SGA 抑制 A-23187 促活性氧生成的 IC₅₀ (1.4 μmol · L⁻¹) 较抑制其促[Ca²⁺]_i 增加的 IC₅₀ (96.2 μmol · L⁻¹) 为低, 说明 SGA 的此两种作用机制不同。A-23187 通过 Ca²⁺ 依赖性机制促进 Neu 生成活性氧⁽⁶⁾, 低浓度 SGA 可能抑制了 Ca²⁺ 调控的 A-23187 的活性氧生成诱导过程, 而非影响[Ca²⁺]_i 所致。

高浓度 SGA (> 25.4 μmol · L⁻¹) 刺激 Neu 生成活性氧, 101.5 μmol · L⁻¹ 以上时也明显促进 Neu [Ca²⁺]_i 增加。虽然 SGA 诱导的 [Ca²⁺]_i 增加先于活性氧生成, 且两量-效曲线大致平行(Fig 3), 但促[Ca²⁺]_i 增加的阈下浓度 (25.4 μmol · L⁻¹) 即有强大活性氧生成刺激作用, 说明 SGA 的活性氧生成刺激作用并非 [Ca²⁺]_i 增加引起, 可能是激活了受细胞内同一机制调控的两平行变化的生化过程。

SGA 预处理 Neu 增强 f-MLP 诱导活性氧生成的作用而抑制其促[Ca²⁺]_i 升高的作用, 和文献⁽⁷⁻¹¹⁾报道的其它 Neu 活化剂 PMA 和 Con A 的作用相似, 但作用机理不清。

A-23187 是个脂溶性复合物, 经非生理性流入机制促进 Ca²⁺ 顺浓度梯度跨膜内流⁽¹¹⁾, f-MLP 诱导的[Ca²⁺]_i 增加属细胞内贮钙释放引起⁽⁷⁾, 促[Ca²⁺]_i 增加或不增加浓度的 SGA 均抑制此两种刺激剂诱导的 Neu [Ca²⁺]_i 增加, 这说明 SGA 对 Neu [Ca²⁺]_i 的影响作用机理复杂, 尚待更多的资料加以证实。

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