Phosphate Buffered Saline Containing Calcium and Magnesium Elicits Increased Secretion of Interleukin-1 Receptor Antagonist

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Abstract

Objective: Phosphate buffered saline (PBS) solutions are commonly used in laboratories for dilutions, washing cell suspensions, and rinsing, as well as additives to cell culture media. In the present study, we evaluated pro- and anti-inflammatory cytokine secretion of peripheral blood mononuclear cells (PBMCs) incubated in medium containing different PBS solutions.

Methods: Human PBMCs were incubated in cell culture medium with different concentrations of PBS containing calcium (Ca^{++}) and magnesium (Mg^{++}) (+/+ PBS). Cells in medium alone or in suspensions containing PBS without Ca^{++} and Mg^{++} (–/– PBS) served as controls.

Results: A dose-dependent increase of interleukin-1 receptor antagonist was found when PBMCs were cultured in medium supplemented with increasing concentrations of +/+ PBS. No significant differences were observed for interleukin-1ß, interleukin-4, interleukin-10, or transforming growth factor ß.

Conclusions: The release of the anti-inflammatory cytokine interleukin-1 receptor antagonist in addition to unchanged levels of pro-inflammatory mediators suggests an important modulatory mechanism of heightened extracellular calcium levels.

Phosphate buffered saline (PBS) solutions are commonly used in laboratory protocols for dilutions, washing cell suspensions, and rinsing culture flasks and plates, as well as additives to cell culture media.[1-6] Phosphate buffered saline is commercially available in different formulations with calcium and magnesium (+/+ PBS) or without (–/– PBS) (**Table 1**).

Calcium functions as a major intracellular second messenger in many signal transduction pathways and triggers a broad range of cellular actions such as muscle contractility, hormone secretion, and vascular tone adjustment.[7] Furthermore, it was shown to influence cellular interactions in sepsis[8] and tissue inflammation.[9] Hotchkiss and colleagues described the impact of calcium on the production of a variety of cytokines such as tumor necrosis factor alpha and interleukin-1 beta (IL-1ß),[10] both involved in the pathophysiology of sepsis. As heightened extracellular calcium concentrations can initiate an inflammatory cascade, we hypothesized whether the addition of –/– or +/+ PBS might have an influence on human peripheral blood mononuclear cells (PBMCs) under different culture conditions. These alterations of extracellular conditions might influence several functions including secretion of cytokines, proliferative responses, and cell death.[11]

It has been recently shown that monocyte-macrophage cell lines can detect increasing extracellular calcium concentrations via the G-protein coupled calcium sensing receptor resulting in increased cytokine secretion (eg, transforming growth factor ß [TGF-ß]).[12,13] In addition, a similar response has been described in human keratinocytes. Bigler and colleagues observed heightened intracellular levels of interleukin-1 receptor antagonist (IL-1ra) when keratinocytes were exposed to increasing levels of extracellular calcium.[14] Binding of IL-1ra to the IL-1 receptor is thought to competitively inhibit IL-1 activity by binding to the IL-1 receptor without cellular activation. This data implies that induction of cytokine secretion (eg, IL-1ra) seems to be closely related to altered extracellular calcium levels.

Therefore, we planned to evaluate if the application of +/+ PBS had an impact on IL-1ß or IL-1ra secretion by human PBMCs. Further, we sought to investigate other anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10),[15,16] or TGF-ß.

Table 1_Phosphate Buffered Saline (PBS) Formulations

	–/– PBS		+/+ PBS	
	Concentration (mg/L)	Molarity	Concentration (mg/L)	Molarity
Calcium chloride ($CaCl_2$)	–	–	100	0.900901
Magnesium chloride ($MgCl_2 \bullet 6H_2O$)	–	–	100	0.492611
Potassium chloride (KCl)	200	2.666667	200	2.666667
Potassium phosphate monobasic (KH_2PO_4)	200	1.470588	200	1.470588
Sodium chloride (NaCl)	8,000	137.93103	8,000	137.93103
Sodium phosphate dibasic ($Na_2HPO_4 \bullet 7H_2O$)	2,160	8.059702	2,160	8.059702

Formulations of phosphate buffered saline solutions (data supplied by Invitrogen Inc., USA)

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Methods

Study Design

This study was conducted at the research laboratory of the Department of Cardiac and Thoracic Surgery (Medical University Vienna) according to the principles of the Helsinki Declaration and Good Clinical Practice and was approved by local authorities. Informed consent was obtained from all participants in this study. Major inclusion criteria were male or female, age 18 to 30 years, body mass index of 18 to 28 kg/m^2, no intake of anti-inflammatory drugs during the last 2 weeks, no acute infection during the last month, no chronic inflammatory disease, and no physical activity prior to testing.

Separation of Peripheral Blood Mononuclear Cells

Venous blood was drawn from healthy young volunteers (n=7). Anticoagulated blood specimens were processed immediately and PBMCs were separated by Ficoll density gradient centrifugation. Buffy coats with mononuclear cells were obtained, washed in Hanks balanced salt solution (HBSS), and resuspended in fresh RPMI 1640 medium (Invitrogen, Austria) supplemented with Glutamax (Invitrogen). Cell concentrations were determined on a Sysmex automated cell counter (Sysmex, USA).

Cell Culture

PBMCs were seeded on 24-well cell culture plates (Perkin Elmer, Austria) at a concentration of 1 × 10^6 per mL, containing 20% of either PBS (Invitrogen) with (+/+ PBS) or without calcium and magnesium (–/– PBS). For further investigations, increasing concentrations of +/+ PBS were added. Dilutions of 1.25×, 1.5×, and 2× +/+ PBS were generated from a stock solution of 10× +/+ PBS. Final molar concentrations for calcium and magnesium in medium supplemented with increasing dilutions of +/+ PBS were as follows: 1× +/+ PBS 0.50 mM Ca^{++}, 0.50 mM Mg^{++}, 1.25× +/+ PBS 0.64 mM Ca^{++}, 0.52 mM Mg^{++}, 1.5× +/+ PBS 0.69 mM Ca^{++}, 0.55 mM Mg^{++}, 2× +/+ PBS 0.78 mM Ca^{++}, 0.60 mM Mg^{++}. Autologous EDTA plasma was used to block Ca^{++} dependent effects. In the control group, volumes were adjusted by adding fresh RMPI 1640 medium. Cell-free supernatants were collected by centrifugation after a culture period of 30 min, 1 h, 2 h, 3 h, and 24 h in an incubator at 37°C and kept frozen at –20°C until further testing.

ELISA Evaluation of Cell Culture Supernatants

Supernatant levels of cytokines were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits for the quantification of IL-1ra, IL-1ß, IL-4, IL-10, and TGF-ß (Duoset; R&D Systems, USA) according to the manufacturer's protocol. In short, 96-well microtiter plates were coated overnight at room temperature with the appropriate capture antibody. After blocking of plates, samples of plasma, supernatants, and standard protein were added to the wells. After a washing step, a biotin-labeled antibody was added to each well and incubated for 2 h. Plates were washed and streptavidin-horseradish peroxidase was added. Color reaction was achieved using tetramethylbenzidine (TMB; Sigma Aldrich, USA) and was stopped by a sulphuric acid stop solution (Merck, Germany). Optical density values were measured at 450 nm on an ELISA plate reader (Victor3 Multilabel plate reader, Perkin Elmer). The intra-assay coefficient of variation (CV) for IL-1ra was 4.6%; for IL-1ß, 4.75%; for IL-4, 6.2%; for IL-10, 4.6%; and for TGF-ß, 2.4%. Minimum detectable doses for IL-1ra were 2.15 pg/mL; for IL-1ß, 1.0 pg/mL; for IL-4, 10.0 pg/mL; for IL-10, 3.9 pg/mL; and for TGF-ß, 4.6 pg/mL.

Statistical Methods

Statistical analyses were performed using Graph Prism 5 software (GraphPad Software, USA). Data is given as mean ± standard error of the mean (SEM). Two-sided Student's *t*-tests for paired comparisons were used to calculate significances. Bonferroni-Holm correction was used to adjust obtained *P* values for multiple testing. A *P* value <0.05 was considered statistically significant.

Results

Cell culture supernatants obtained from PBMC after 24 h evidenced IL-1ra levels of 388.3 ± 51.5 pg/mL in the control group (medium) and 276.8 ± 39.0 pg/mL in wells supplemented with PBS without calcium and magnesium. When adding PBS containing both calcium and magnesium to cell cultures, this value increased to 636.7 ± 61.3 pg/mL. This effect could be totally abrogated by adding autologous EDTA containing plasma, as evidenced by values of 27.1 ± 15.9 pg/mL (**Figure 1**).

Since the addition of +/+ PBS induced an increment of IL-1ra levels, we also tested for other pro- and anti-inflammatory cytokines (IL-1ß, IL-4, IL-10, and TGF-ß). No changes were found for IL-1ß; values ranged below or slightly above detection limit (0 to 8.3 pg/mL). Similar results were obtained when analyzing PBMC-derived cell culture supernatants for anti-inflammatory cytokines. No major differences were detected either for IL-4, IL-10, or TGF-ß.

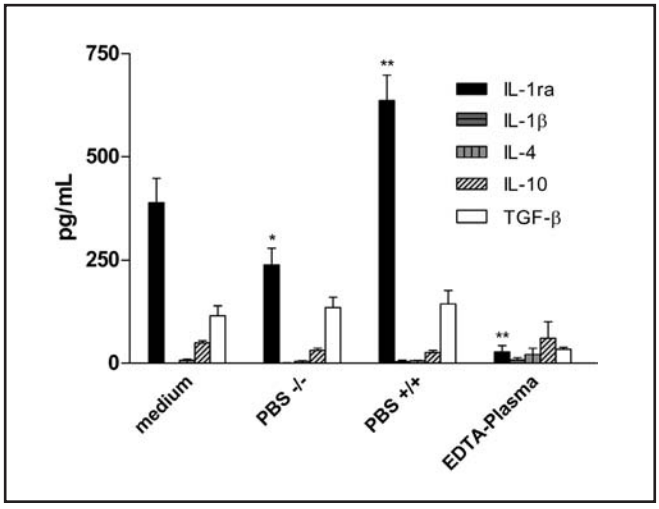

Figure 1_Cytokine levels of IL-1ra, IL-1ß, IL-4, and TGF-ß in supernatants from PBMCs collected after 24 h. Adding calcium- and magnesium-containing PBS to cell cultures increases values of IL-1ra, whereas addition of EDTA-plasma leads to a strong reduction of cytokine release. *P< 0.05, ** P < 0.01 versus medium.

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To further prove our data, we performed the same set of experiments and added increasing doses of calcium- and magnesium-containing PBS to PBMC cultures. We found a dose-dependent increment in IL-1ra supernatant levels (levels of 636.7 ± 61.3 pg/mL at 1× +/+ PBS, 1282.3 ± 351.2 pg/mL at 1.25× +/+ PBS, 1439.6 ± 205.5 pg/mL at 1.5× PBS, and 1,908.8 ± 46.4 pg/mL at 2× +/+ PBS. No dose-dependency was observed for PBMC cell culture supernatants analyzed for IL-1ß, IL-4, IL-10, or TGF-ß (**Figure 2**).

We also found no significant differences in IL-1ra levels after incubation periods of 30, 60, 120, and 180 min, thus suggesting that a prolonged time of exposure to heightened extracellular ion levels during cell culture is required to influence cytokine secretion (**Figure 3**).

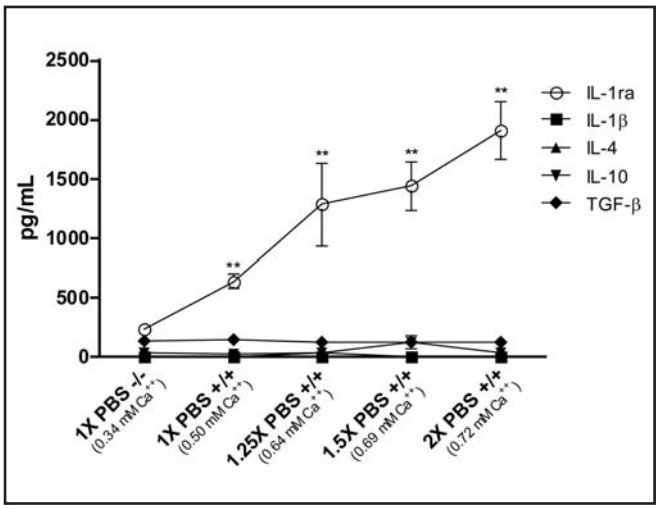

Figure 2_Dose-dependent effect of supplementing PBMC cell cultures with increasing concentrations of calcium- and magnesium-containing PBS, thus resulting in heightened levels of IL-1 receptor antagonist. **P* <0.05, ***P* <0.01 versus PBS without calcium and magnesium.

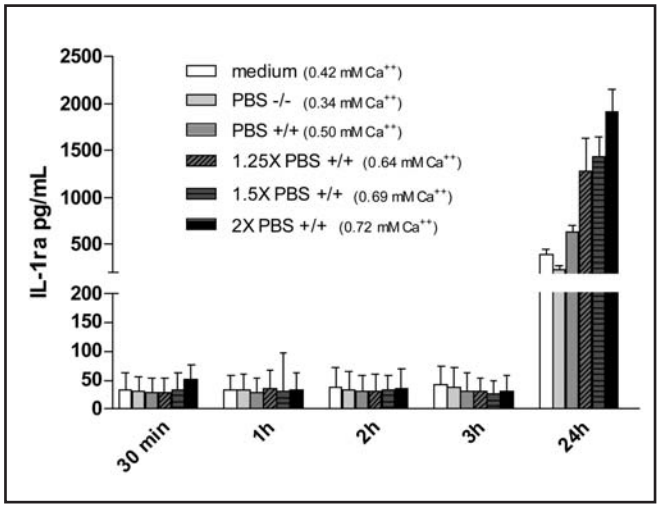

Figure 3_Short-term responses of IL-1ra secretion by human PBMC after incubation times of 30 min to 3 h. No significant differences were observed between groups at short incubation intervals. Heightened extracellular ion concentrations in medium containing increasing levels of +/+ PBS seem to have an influence on cellular IL-1ra release only after prolonged exposure times.

Discussion

In this present study, we provide evidence of a significant effect of PBS containing calcium and magnesium on cell cultures of human PBMCs. Because PBS either in –/– or +/+ formulations is used ubiquitously in laboratories, cellular responses induced after contact or co-incubation with these solutions are evident. In support of this hypothesis, addition of EDTA-plasma significantly abrogates cytokine secretion, in particular of IL-1ra. As EDTA complexes free Ca^{++} ions and therefore inhibit calcium-dependent cellular functions,[17] we believe that the effects seen by +/+ PBS are Ca^{++} triggered and cause interactions which can initiate increases in cytokine release.

Increasing extracellular calcium gradients were shown to play an integral part in inflammatory cell recruitment to sites of infection and apoptosis. This mechanism might well be of importance in in vitro conditions. Interestingly, in contrast to the described pro-inflammatory effect of Ca^{++}, we found a dose-dependent relationship with the secretion of an anti-inflammatory mediator (IL-1ra) in vitro. IL-1ra, predominantly secreted by monocytes,[18-19] interferes with the induction of a pro-inflammatory state by inhibiting the cellular interactions of IL-1 on many cell types such T-, B-, and mononuclear cells.

Since, during most laboratory applications, cells are exposed to PBS solutions for rather short intervals (eg, minutes and hours), we tested the impact of +/+ PBS in a time-course experiment. No changes of IL-1ra levels were detected within the first 3 h of incubation.

These results indicate that increasing extracellular ion concentrations such as calcium can also mediate anti-inflammatory cellular mechanisms by an initiation of IL-1ra cytokine release; however, further investigations are required to address this question. LM

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