Effects of fixatives on function of pulmonary surfactant

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Bachofen, H., U. Gerber, and S. Schürch. Effects of fixatives on function of pulmonary surfactant. J Appl Physiol 93: 911–916, 2002.—The structure of pulmonary surfactant films remains ill defined. Although plausible film fragments have been imaged by electron microscopy, questions about the significance of the findings and even about the true fixability of surfactant films by the usual fixatives glutaraldehyde (GA), osmium tetroxide (OsO4), and uranyl acetate (UA) have not been settled. We exposed functioning natural surfactant films to fixatives within a captive bubble surfactometer and analyzed the effect of fixatives on surfactant function. The capacity of surfactant to reach near-zero minimum surface tension on film compression was barely impaired after exposure to GA or OsO4. Although neither GA nor OsO4 prevented the surfactant from forming a surface active film, GA increased the equilibrium surface tension to above 30 mN/m, and both GA and OsO4 decreased film stability as seen in the slowly rising minimum surface tension from 1 to 5 mN/m in 10 min. In contrast, the effect of UA seriously impaired surface activity in that both adsorption and minimum surface tension were substantially increased. In conclusion, the fixatives tested in this study are not suitable to fix, i.e., to solidify, surfactant films. Evidently, however, OsO4 and UA may serve as staining agents.

STUDYING RAT LUNGS FIXED BY vascular perfusion, Gil and Weibel (7) were the first to convincingly demonstrate by transmission electron microscopy a thin extracellular lining layer covering the alveolar surface. This layer was composed of two phases, i.e., a liquid base layer and a lamellar superficial layer of (osmiophilic) phospholipids. This structural arrangement appeared to support physiological observations and the hypothesis that the lining layer consists of an aqueous hypophase covered by a monomolecular film of phospholipids (8). However, the osmiophilic film was fragmented and disposed in patches, and, in contradiction to the physiological hypothesis of a monolayer, the morphology of the natural surface film was rather inhomogeneous in that lamellar structures with stacks of three to six osmiophilic layers were observed. By using alternative methods, the postulated continuity of the alveolar lining layer could be demonstrated (5, 27), but the structure of the surfactant film remained ill defined. Improvements to the techniques of lung fixation and staining did not yield more conclusive results. Usually, the best and most “typical” electron micrographs of the osmiophilic lining layer are shown without information about its continuity (26). Our laboratory’s own morphometric analysis of a large series of rabbit lungs fixed by vascular perfusion under well-controlled conditions (4) revealed an osmiophilic layer covering ∼10% of the entire alveolar surface only (unpublished results). Before discarding the hypothesis of a continuous surfactant lining, possible shortcomings and artifacts of the usual fixatives and stains [glutaraldehyde (GA), osmium tetroxide (OsO4), uranyl acetate (UA)] should be considered: 1) It is conceivable that the fixatives induce a sudden and substantial increase in surface tension, resulting in disruptions of the film. 2) Fluorescence light micrographs (12, 15, 29) and atomic force micrographs (1, 9, 30) show that the surfactant film is not homogeneous, and hence fixable and nonfixable parts, such as pure dipalmytoylphosphatidylcholine (DPPC) domains, might coexist. 3) If there is an appropriate mixture between saturated and nonsaturated phospholipids together with proteins, the film could be fixed as a cohesive “skin.” This would imply a complete elimination of surface activity that is eliminating the capacity to reach near-zero surface tensions on film compression. Owing to its delicacy, the film could be fragmented by further processing (embedding and cutting). 4) The surfactant film can be stained but is, by itself, not fixable. However, those parts of the film where the polar groups stick to a fixable hypophase will be preserved. In fact, clearly defined lining layers could be observed on top of protein-rich alveolar fluid (21).

The effects of fixatives on the function and hence the structure-function relationship of surfactant films have not yet been examined. Information about this interplay might give some insight into the problems of film fixation mentioned above. To this aim, the surface activity of natural lung surfactant brought into contact

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with fixatives was tested within a captive bubble surfactometer.

**METHODS**

**Surfactant**

For this study, we used natural bovine surfactant obtained from alveolar lavage material of bovine lungs (32). The preparation contains ~70% phospholipids and ~10% proteins [i.e., ~8% surfactant protein (SP)-A, 1% SP-D, and ~1–1.5% SP-B and SP-C]. For testing, the natural surfactant is suspended in a solution of 0.9% NaCl + 2.5 mM CaCl₂ + 10 mM HEPES (pH 6.9) at a phospholipid concentration of 1 mg/ml. We used a captive-bubble surfactometer to thoroughly test this surfactant, and its high surface activity has been proven (21, 17).

**Fixatives**

The usual fixatives used for lung fixation and preservation of the alveolar lining layer (4, 7, 26, 31), i.e., GA [OHC(CH₂)CHO], OsO₄, and UA [UO₂(OCOCH₃)₂·2H₂O], were either added to the chamber of the surfactometer, in which a film had already been adsorbed to the surface of an air bubble, or mixed with the surfactant suspension before filling the chamber (see Procedures below). Concentrations of fixative solution were chosen to yield final concentration of 2.5% GA (buffered in 0.1 mM Na-cacodylate), 1% OsO₄ (buffered in 0.1 mM Na-cacodylate), and 0.5% UA (in maleate buffer, pH 5) within the surfactometer.

**Surfactometer**

The captive bubble surfactometer has been described in detail elsewhere (19, 20). In short, the instrument consists of a sample chamber cut from cylindrical glass tubing of high quality with an inner diameter of 1 cm. A metal piston with a tight O-ring seal is fitted into the glass tubing from one end. The other end is fitted into a plate of stainless steel, which is provided with an inlet port in the center for adding solutions and the air bubble to the chamber. Chamber and piston are vertically mounted within a sturdy rack of steel whose height is regulated by a micrometer gear with minimal redundancy. For usual measurements, such as the control experiments shown in Fig. 1, the chamber is filled with the surfactant suspension, to which solutes (fixatives) can be added. The chamber content is stirred with a small magnetic bar, and its temperature is maintained at 37°C. A small air bubble is introduced from beneath, whose volume and hence surface area can be changed by compression and decompression brought about by changing the height of the rack.

During the compression-reexpansion cycles, the bubble is continuously recorded on a video recorder. Selected single frames are stored in RAM for later image processing and analysis (18). Bubble areas and volumes are calculated by an original algorithm relating bubble height and diameter to areas of revolution, and the bubble surface tension is determined by using the method of Malcolm and Elliott (11).

**Procedures**

**Exposure of the film to fixatives after adsorption to the bubble surface.** To this aim, a modification of technique was necessary. Instead of directly filling the chamber, the surfactant suspension together with a small air bubble were introduced into a short piece of dialysis tubing consisting of a molecular porous membrane. The tubing (Spectra/Por 4 MWCO: 14.00, The Spectrum Companies, Gardena, CA) was tied up at both ends. It is fully transparent and allows perfect visualization of the bubble size and shape. The inner top end of the tubing was provided with a cap of 1% agarose gel to ensure a smooth and fully hydrophilic contact with the bubble. The filled piece was introduced vertically into the chamber (its volume is about half of that of the chamber), and the remaining chamber volume was filled with a particular fixative. A preliminary test showed a rapid diffusion of the fixatives through the tubing wall. Cotton threads soaked with linolenic acid inside the tubing turned black 10 min after the tubing was submerged in the OsO₄ solution. Five minutes after chamber filling, two cycles of film compression to minimum surface tension and reexpansion were performed, followed by a further compression to minimum surface tension. Thereafter, the bubble volume was kept constant for 1 h, and every 5 min a video image was recorded. Finally, three continuously recorded expansion-compression cycles were carried out.

**Exposure of surfactant to fixatives before film adsorption.** These sets of experiments served to check whether the fixatives exert an inhibitory effect on surfactant adsorption to the surface of the air bubble. To this aim, the surfactant suspension and a particular fixative were mixed, and the mixture was introduced into the chamber together with a small air bubble. Thus the fixatives were in full contact with the surfactant before any film adsorption. After filling the chamber, exactly the same procedure was followed with regard to bubble compression and decompression as described above.

Because the comparative experiments revealed a detrimental effect of the UA solution on surfactant function, additional experiments were done to check the influence of the acid maleate buffer (GA and OsO₄ were buffered with Na-cacodylate, total osmolarity 350 mosM, pH 7.4; UA with maleate buffer, total osmolarity 350 mosM, pH 5).

**RESULTS**

**Procedure A**

Figure 1 shows the results (means ± SD, n = 3) of the control experiments. After film adsorption of the natural surfactant to the bubble surface, the equil-
rium surface tension was $20.9 \pm 2.9 \text{ mN/m}$. On the first compression of the film, a minimum surface tension of $1.2 \pm 0.2 \text{ mN/m}$ was achieved. The minimum surface tensions reached on the second and third compression were equal (within the error of a single measurement of $\pm 0.3 \text{ mN/m}$) to that obtained on the first compression. The area compressions required to compress the film from the equilibrium to the minimum surface tension were between 17 and 18% for both the initial and the final compression. Under constant compression during 1 h, there was a slight but continuous increase in surface tension to $5.1 \pm 0.8 \text{ mN/m}$. The final decompression-compression cycles again achieved very low surface tensions of $1.0 \pm 0.3 \text{ mN/m}$, demonstrating an unimpaired surface activity of this surfactant.

The effects of the fixatives GA, OsO₄, and UA on surfactant function after film adsorption (the bubble being placed in the dialysis tubing) are shown in Fig. 2. For all three fixatives, the equilibrium surface tensions are slightly higher than in the control experiments. With regard to the further effects of the fixatives on the film, there was a considerable difference between GA and OsO₄ on the one side and UA on the other. Films exposed to GA and OsO₄ could be compressed to very low surface tensions comparable to those observed in the control experiments. However, under constant compression (constant bubble volume) for 1 h, the stability of films appeared to be impaired. With GA, a spontaneous increase of surface tension from $\sim 1 \text{ mN/m}$ to $10.8 \pm 7.4 \text{ mN/m}$ could be observed. With OsO₄, this increase amounted to $17.2 \pm 5.3 \text{ mN/m}$. Despite this apparent loss of stability, the surface activity of the film was preserved as shown by the final decompression-compression cycles, by which a minimum surface tension close to zero could easily be achieved with film area compressions of $\lesssim 20\%$. In contrast, UA induced a seriously impaired surface activity. The minimum surface tension was high ($17.8 \pm 4.6 \text{ mN/m}$) followed by a spontaneous increase during the next hour to $28.2 \pm 12.5 \text{ mN/m}$. Remarkably, the final decompression-compression cycles revealed a residual capacity to lower the surface tension, albeit the minimum surface tension was much higher than those obtained in the experiments with added OsO₄ or GA to the surfactant. The control experiments (results shown in Fig. 1) were performed with the use of the same tubing design.

**Procedure B**

The effects of fixatives mixed with the surfactant suspension, i.e., before adsorption of a surface film, are illustrated in Fig. 3. GA appears to interfere with film adsorption, in that the equilibrium surface tension assumes a high value of $\sim 50 \text{ mN/m}$. On compression, the film resumes a high surface activity, and already after the second compression cycle the minimum surface tension was close to zero, but the film area compressions required to reach these low minimum surface tensions were $\sim 60\%$, reflecting the relatively high equilibrium surface tension of $\sim 50 \text{ mN/m}$. The compressed film was less stable than in the tubing experiments. After the third compression, a rapid spontaneous increase to a plateau surface tension of $\sim 15 \text{ mN/m}$ occurred. Similar alterations are caused by OsO₄. Remarkably, however, the equilibrium tension was less affected by OsO₄ than by GA because the equilibrium surface tensions for the samples with OsO₄ were $\sim 32 \text{ mN/m}$, close to those of the control values. The associated film-area compressions required to reach near-zero minimum surface tensions were between 40 and 50%. In contrast, the addition of UA to the surfactant suspension has a highly deleterious effect on surfactant function. After the adsorption time of 5 min, the equilibrium surface tension was higher than $70 \text{ mN/m}$, which is about equivalent to that of pure water. Compression of the bubble revealed some surface activity, but the minimum surface tension remained high ($\gtrsim 30 \text{ mN/m}$). The large standard deviation, however, reflects the erratic behavior of this preparation. Finally, Fig. 1
also shows that the acid maleate buffer of the UA solution (pH 5) does not interfere with surfactant function, because the surfactant function is virtually identical to that in the control experiments using HEPES-buffered saline. Thus UA is the true inhibitor.

**DISCUSSION**

Preceding the actual discussion, comments pertaining to the methodological procedures are appropriate. We used two methods of exposing the surfactant to the fixatives. In procedure B, the fixative and the surfactant were mixed, and the mixture was introduced to the bubble chamber. An air bubble was then introduced into the chamber. In procedure A, film formation by the surfactant by adsorption was independent of the fixatives. A normal film was formed by adsorption of the surfactant material at the bubble surface. The dialysis tubing that contained the bubble and the surfactant was exposed to the fixative on filling of the remaining space in the chamber of the captive bubble surfactometer with the fixative.

It has been shown that the method of choice for the visualization of surfactant films in situ is the fixation of lungs by vascular perfusion with fixatives (2, 7). To simulate a comparable situation in vitro, we designed the tubing setup as described in METHODS (procedure A). Preliminary experiments (not shown here) and the control experiments (Fig. 1) demonstrated the feasibility of this procedure. In particular, the tubing wall did not introduce appreciable optical problems. As an alternative, a hypophase exchange technique might have been used. However, this technique requires a successive washout of at least four times. This would have introduced problems with timing. In addition, the manipulation with highly toxic fumes close to the face of the operator is a health hazard that cannot be justified.

The functional properties of natural and lipid-extract surfactant have been extensively examined and analyzed in vitro and in situ (4, 15, 17, 21). Interpretation of the findings is hampered by the ill-defined structure of the film, i.e., by a fragmentary knowledge of the structure-function relationship (31). More recent techniques made it feasible to image native films in vitro (1, 9, 12, 15, 22, 29, 30). These studies indicate a high complexity of the structure of the film and discard the widely accepted view of a putative homogeneous monolayer. However, for in situ imaging, chemical fixation and staining of the film for electron microscopy is the only feasible technique. As mentioned in the introduction, the results are not fully convincing, presumably because of persisting problems of film preservation, fixation, and staining. GA and OsO 4 are the most widely used fixatives. Other chemicals, including UA, tannic acid, and potassium permanganate have been used as staining agents. Knowledge of the chemistry of the fixation processes and the resulting alterations at the molecular level is rather scant. There is good evidence that GA exerts its fixative effect by cross-linking of proteins; the solidification appears to be dependent on the concentration of GA and the type and concentrations of proteins (10). For example, it has been shown that for fixing an albumin solution a minimum albumin concentration of 3% (per mass) is required (3). Films of natural surfactant contain ~10% proteins, ~1.0–1.5% of which constitute the small hydrophobic surfactant proteins SP-B and SP-C. Most of the surfactant protein is made up of the large hydrophilic SP-A and some SP-D. The latter does not seem to possess any activity related to surface activity (28) and hence will not be considered in our discussion. Because the distribution of proteins is inhomogeneous within the film [proteins essentially are restricted to liquid expanded domains (14)], a partial fixation by GA appears to be feasible. However, information about the fixability of surfactant-associated proteins with their particular structure is not available.

SP-B plays the major role in adsorption of new material into the air-liquid interface and is important in a number of activities related to the surface tension properties, among them the stabilization of the air-water lipid film. In contrast to SP-B, there are only a few activities described for SP-C, which largely overlap with the activity of SP-B (28). SP-C also promotes lipid adsorption into the air-liquid interface and stabilizes the lipid film. In addition, SP-C plays a substantial role in the surfactant reservoir formation (28). Both proteins appear to be important for the selective enrichment of the surface film on adsorption as well as on compression (13, 23, 28). SP-A can also promote phospholipid adsorption, but the effect is weak (17). In our experiments, the effects of GA and OsO 4 on the surface activity of previously adsorbed film is relatively minor, as only the long-term stability is impaired. The surface activity (the capacity to lower the surface tension) can be restored as shown by the final expansion-compression cycles.

As a strong oxidant, OsO 4 reacts with double bonds in the hydrocarbon chains of fatty acids. However, OsO 4 does not fix saturated phospholipids such as DPPC (6, 25). Because natural surfactant films contain both saturated and unsaturated phospholipids, only a partial fixation can be expected, which might result in a defective imaging of the molecular structure of the film. UA is considered as a staining agent that binds specifically to phosphate groups of phospholipids in lipid bilayers and thus increases membrane stability. However, UA also has a fixative effect as shown by additional solidification of lung tissue after previous fixation with GA and OsO 4 (2).

Considering these facts, one would barely expect the usual fixatives to be promising agents for the preservation of flimsy structures such as surfactant films. Indeed, this surmise is supported by the present experiments. Fixation implies a loss of surface activity, and, evidently, this is not the case. After a functional film has been adsorbed to the air-liquid interface of the bubble in the surfactometer, the contact with GA or OsO 4 does not eliminate the surface activity of the film. The film can be compressed in a repetitive manner to very low surface tensions close to zero; the only observed functional alteration is a moderate impairment.
of the stability of compressed films because the minimum surface tension stayed below 5 mN/m during an initial period of 1 min at constant bubble volume. Interestingly, both GA and OsO₄ have a quite similar effect, although their putative mechanisms of disturbance are different. One might speculate that GA and OsO₄ derange “nonessential” components of the film but leave the most important component, i.e., DPPC, unaffected. More specifically, GA might reduce the film stability by its effect on SP-B and SP-C, whereas OsO₄ might slightly disrupt lipid chain packing in the surface film by binding to the unsaturated phospholipid chains. However, we are not able to distinguish between the two mechanisms, because there were no discernible differences in minimum surface tension nor in film area compressions needed to achieve near-zero tensions. The effect of the “stain” UA, on the other hand, has a far more deleterious effect on surfactant function: after contact with UA, the minimum surface tension is increased far above the level required for stabilization of alveoli. However, a considerable surface activity persists, which indicates that UA induces a serious disturbance rather than a fixation of the film. Possibly, UA molecules bound to the polar ends of phospholipids act as impurities that cannot be “squeezed-out” by film compression.

The direct mixing of fixatives with the surfactant suspension, i.e., the exposure of surfactant to the fixatives GA and OsO₄ before film adsorption to the bubble’s surface, has a similar but more pronounced effect. Film formation in the presence of GA is impaired in that the equilibrium surface tension is ~50 mN/m. Although minimum surface tensions of 1–2 mN/m can be achieved, the film area compressions necessary are ~60%. The effect of OsO₄ is less severe, as the equilibrium surface tension is ~30 mN/m and the film area compressions to reach near-zero surface tension are ~50%. It is likely that the high-equilibrium surface tension of 50 mN/m in the presence of GA is due to its cross-linking effect on the surfactant proteins (mostly SP-B and SP-C), because these two proteins promote film formation by lipid adsorption. OsO₄ appears to have a minor effect on the ability of SP-B or SP-C to promote the adsorption of a film with high surface activity. However, film purification on compression is impaired as seen by the relatively large area compressions required to reach near-zero surface tensions. In contrast, the effect of UA is detrimental. The initial equilibrium tension is close to that of a pure air-water interface, suggesting a complete inhibition of film formation by UA. The decrease in surface tension on compression of the bubble does not imply a secondary formation to a coherent film as the surface tension remains very high.

Thus the results of these experiments show that the fixatives GA and OsO₄ do disturb but do not abolish the surface activity of films of natural surfactant. By inference, they do not truly fix or solidify, respectively, surfactant films as isolated structures on top of an aqueous hypophase. Interestingly, the most detrimental agent is UA, which is not required for but appears to improve the visualization of surfactant films by transmission electron microscopy. Returning to the possible shortcomings of fixation of surfactant films mentioned in the introduction, one can discard mechanisms 1) and 3) in view of the residual surface activity of the films (Fig. 2). Neither a disrupted film nor a cohesive “skin” could likely cause changes in surface-tension as observed during the last expansion-compression cycle. Mechanism 1) also appears improbable. The fractional fixation of countless domains would introduce a huge amount of impurities into the film with the consequence of a seriously impaired surface activity. On the contrary, possibility 4) is most attractive. In normal lungs, surfactant films can best be observed at sites of an appreciable, proteinaceous hypophase, i.e., in alveolar corners, and the most convincing images of coherent films can be obtained from lungs with discrete, proteinaceous pulmonary edema (3). This observation might show the way for an improved technique for preservation of surfactant films in vitro and thus to study their structure-function relationship. A fixable hypophase has to be found on which surfactant films can be spread or adsorbed and which does not interfere with the function of the surfactant. Numerous attempts have been made in this direction. It appears that a sodium alginate solution, which can be solidified by adding calcium ions (16), is a promising method to this purpose (22).

A remarkable by-product of this study is the fact that the surface activity of lung surfactant is only slightly impaired by the aggressive oxidant OsO₄. Hence, the surfactant film as the first contact surface area in the lung might also be rather resistant to inhaled oxidants of polluted air and oxidative fumes. With regard to acute oxidant injuries of the lung, the weak link in the chain is probably the blood-gas barrier. By increasing barrier permeability, which results in exudation of plasma components and inflammatory products, oxidants indirectly inhibit surfactant function (24).

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